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# Dynamic changes in the localization of synapse associated proteins during development and differentiation of the mammalian retina

Mary Heather West Greenlee  
*Iowa State University*

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**Dynamic changes in the localization of synapse associated proteins during  
development and differentiation of the mammalian retina**

**by**

**Mary Heather West Greenlee**

**A dissertation submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of  
DOCTOR OF PHILOSOPHY**

**Co-Majors: Neuroscience; Zoology (Neurobiology)**

**Major Professors: Don Sakaguchi and Philip Haydon**

**Iowa State University**

**Ames, Iowa**

**1999**

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**~~Committee Member~~**

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**Committee Member**

Signature was redacted for privacy.

**Committee Member**

Signature was redacted for privacy.

**Co-major Professor**

Signature was redacted for privacy.

**Co-major Professor**

Signature was redacted for privacy.

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**For the Co-major Program**

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## GENERAL INTRODUCTION

The proper function of our nervous system is dependent upon the appropriate formation of millions of synapses. Understanding the processes which guide the formation and refinement of synaptic contacts is a crucial issue in neurobiology. Refinement of synaptic contacts during development has been extensively studied in the visual system (Shatz, 1996). Visual system development is dependent upon appropriate formation of the retina, its target structures in the brain, higher visual centers and the connections that are formed between them (see Man-Kit Lam and Shatz, 1991). In addition, activity dependent refinement fine tunes these connections such that our brain receives a precise representation of the visual world around us (Shatz, 1996).

The vertebrate retina is a highly organized structure with a characteristic laminar organization. It is composed of three cellular layers separated by two synaptic layers (Rodiek, 1973). The outermost cellular layer, the outer nuclear layer (ONL) contains cell bodies of photoreceptors, the cells which transduce light stimulus to neural output. In the outer synaptic lamina, the outer plexiform layer (OPL), photoreceptors synapse with bipolar cells whose cell bodies are in the inner nuclear layer (INL). These bipolar cells project to the inner plexiform layer (IPL) where they synapse with retinal ganglion cells, whose cell bodies form the ganglion cell layer (GCL). Axons of retinal ganglion cells in the optic fiber layer (OFL) project and relay the signal to principal relay centers within the brain. In addition to this "through pathway", horizontal cells and amacrine cells in the INL, form lateral connections and synapse in the OPL and IPL respectively. These lateral connections provide some visual processing at the level of the retina, and contribute to specific receptive field properties on individual retinal ganglion cells (Masland, 1996). In addition, two types of glial cells are present in the vertebrate retina. Müller cells are radial glial cells with their cell bodies in the INL, derived from retinal neural epithelium (Polley et al., 1989). Astrocytes are found along the inside of



the optic cup with somata and processes principally within the OFL. Astrocytes are not born in the retina, instead, they migrate into the retina via the optic nerve (Watanabe and Raff, 1988).

Within the organized structure of the retina, there is considerable synaptic diversity. Photoreceptors and bipolar cells form ribbon synapses (Sjöström, 1953; Kidd, 1962). Ribbon synapses are morphologically distinct from conventional type synapses found throughout most of the nervous system. Amacrine cells make synaptic contacts via conventional synapses (Mandell *et al.*, 1990). Finally, horizontal cells release their neurotransmitter from non-vesicular synapses (Schwartz, 1987).

The five retinal cell types (photoreceptors, horizontal, bipolar, amacrine and ganglion cells) can be further divided into subtypes. There are two basic types of photoreceptors, rods and cones. The cones can be further subdivided according to what wavelength of light they absorb (Dowling, 1987). There are two types of horizontal cells, classified as A and B by morphological criteria (Polley *et al.*, 1989). Bipolar cells are subdivided into ON or OFF bipolars, based on whether they depolarize or hyper polarize in response to glutamate. ON bipolars have their terminals in the inner 3/5 of the IPL and OFF bipolars have their terminals in the outer 2/5 of the IPL. Ganglion cells are divided based on soma size and can further be subdivided into either ON or OFF based on their response to light. ON ganglion cells synapse with ON bipolar cells in the inner 3/5 of the IPL, and OFF ganglion cells synapse with OFF bipolar cells in the outer 2/5 of the IPL (Dowling, 1987). The amacrine cells make the greatest contribution to the cellular diversity of the mammalian retina (Masland, 1996). Amacrine cells are subdivided biochemically, based on their primary neurotransmitter (Pourcho, 1996). These biochemically distinct cells have their processes in a characteristic organization within the IPL. This organization has led to the descriptive division of the IPL into five sub-lamina. Sub-lamina 1 and 2 make up the outer 2/5 of the IPL, and sub-lamina 3-5 make up the inner 3/5 (Karten and Brecha, 1983; Marc, 1986).

The development of the vertebrate retina follows a very characteristic pattern. The retina begins as an out pocketing of the neural tube. When this eye bud contacts presumptive head ectoderm it induces the formation of the lens, which in turn causes invagination of the optic vesicle, forming a concave, bi-layer arrangement of neural epithelium. The layer closest to the lens will become retina, and the layer closest to the developing brain will become retinal pigment epithelium . All of the cell types in the retina arise from the neural epithelial progenitors in this primitive eye cup. Extensive birthdating studies, have demonstrated a clear pattern of cell genesis in the developing mammalian retina (Polley *et al.*, 1989).. The earliest cells to leave the mitotic cycle differentiate into ganglion cells. The next cohort of cells to exit the mitotic cycle are cones, horizontal and amacrine cells. Rods, bipolars, and another subclass of horizontal cells exit the mitotic cycle later. The last cells to exit the mitotic cycle are Müller cells, radial glial cells found in the retina which are from the same progenitors as retinal neurons (Polley *et al.*, 1989).

The synaptic development of the vertebrate retina also proceeds in a characteristic manner. Synaptic profiles of conventional type synapses are first observed in the inner plexiform layer, presumably amacrine cells synapsing with ganglion cells and with each other. The ribbon synapses of photoreceptors in the outer plexiform layer are the next synapses to develop. The latest synapses to form are those which connect the outer retina to the inner retina, the ribbon synapses of the bipolar cells in the IPL (Weidman and Kuwabara, 1968).

Another feature of retinal development is correlated "waves" of spontaneous, bursting activity in ganglion and amacrine cells (Feller *et al.*, 1996; Meister *et al.*, 1991). Spontaneous activity is present prior to eye opening, and in many mammals, prior to birth (Galli and Maffei, 1988; Shatz, 1996). These waves are dependent on cholinergic neurotransmission, and depending on retinal maturity, can be modulated by GABA and glutamate (Feller *et al.*, 1996; Fisher *et al.*, 1998).

During nervous system development, activity is required for synaptic refinement and establishment of proper circuitry. In the developing retina, blocking cholinergic dependent spontaneous activity resulted in an abnormal anatomical and physiological expansion of retinal ganglion cell receptive fields (Sernagor and Grzywacz, 1996). Segregation of developing retinal ganglion cell dendrites into ON and OFF sublamina in the IPL is dependent on glutamate mediated synaptic transmission (Bodnarenko *et al.*, 1995). In the lateral geniculate nucleus (LGN) the principal visual relay center in the brain, ipsilateral and contralateral retinal ganglion cells inputs are segregated into separate lamina. This segregation is the result of refinement of initially diffuse ganglion cell terminals (Shatz and Stryker, 1988), and requires, cholinergic-dependent spontaneous waves of activity in the retina (Penn *et al.*, 1998). The only cholinergic neurons in the retina are a subclass of amacrine cell, the starburst amacrine cell. Cholinergic amacrine cells can be identified histochemically with an antibody against choline acetyltransferase (ChAT), the enzyme which makes acetylcholine (Feller *et al.*, 1996; Tucek, 1976; West Greenlee *et al.*, 1998). Cholinergic, starburst amacrine cells exist as two mirror symmetric sub-populations, one with their somata in the INL, and their processes in sublamina 2 of the IPL, and a "displaced" population with their cell bodies in the ganglion cell layer and their processes in sublamina 4 of the IPL (Famiglietti, 1983).

Propagation of spontaneous activity in the developing retina is dependent upon synaptic transmission (Feller *et al.*, 1996). An integral part of chemical synaptic transmission is vesicular release of neurotransmitter from the presynaptic neuron into the synaptic cleft. Release of neurotransmitter takes place via fusion of small synaptic vesicles containing neurotransmitter, with the presynaptic terminal membrane. Docking and fusion of synaptic vesicles is a highly regulated process (Calakos and Scheller, 1996). One model to explain the regulation and the specificity of neurotransmitter release is the SNARE hypothesis (Sollner *et al.*, 1993). In this model, Syntaxin (Bennett, 1995) and Synaptosomal protein of 25 kDa (SNAP-25) (Oyler *et al.*, 1989), two t-SNAREs present on the target membrane, form a

binding site for Synaptobrevin of Vesicle Associated membrane protein (VAMP) (Baumert *et al.*, 1989), the v-SNARE, present on the synaptic vesicle. The interaction of these three proteins forms the SNARE complex. This SNARE complex then serves as a binding site for cytosolic proteins alpha-SNAP and N-ethylmaleimide-sensitive factor (NSF). NSF then hydrolyzes ATP and primes the vesicle for fusion upon an increase in local intracellular  $Ca^{++}$  concentration (Calakos and Scheller, 1996). Interaction of other membrane bound proteins with components of the SNARE complex can prevent formation of the SNARE complex and thereby provides multiple mechanisms for regulation of the release process. For example Synaptophysin (p38) a synaptic vesicle protein, binds to VAMP and may prevent formation of the SNARE complex (Edelmann *et al.*, 1995). Synaptotagmin (p65) is also a synaptic vesicle protein and interacts with Syntaxin. Association of Synaptotagmin with Syntaxin prevents binding of alpha-SNAP with the SNARE complex, thereby perhaps regulating vesicle fusion (Bennett *et al.*, 1995). Synaptic vesicle proteins can also affect transmitter release prior to formation of the SNARE complex. Rab3A for example may be involved in targeting of synaptic vesicles to the presynaptic terminal (Ngsee *et al.*, 1993).

Functional studies of SNARE complex proteins and their role in transmitter release have been greatly facilitated by the use of Clostridial neurotoxins (Niemann *et al.*, 1994). These toxins, tetanus toxin and the seven serotypes of botulinum toxin, inhibit neurotransmitter release by specifically cleaving one of the three members of the SNARE complex (Hayashi *et al.*, 1994). The toxins exist as a dimer of a heavy chain and a light chain. The heavy chain binds to a receptor on the extracellular surface of the neuron, and the protein is internalized. Once inside the cell, the light chain is a very stable, long acting zinc-dependent protease (Ahnert-Hilger and Bigalke, 1995). SNAP-25 is cleaved by botulinum toxins A and E (Schiavo *et al.*, 1993), Syntaxin is cleaved by botulinum toxin C (Igarashi *et al.*, 1996), and Synaptobrevin is cleaved by botulinum toxins B,D,F, G and tetanus toxin (Ferro-Novick and Jahn, 1994).

While a SNARE complex appears to be ubiquitous in all presynaptic terminals, many other presynaptic terminal associated proteins are heterologously distributed among morphologically (Catsicas *et al.*, 1992; Hayashi *et al.*, 1994; Morgans *et al.*, 1996) and biochemically (Mandell *et al.*, 1992) distinct synapses. This is often accomplished via differential expression of protein isoforms (Bachman and Balkema, 1993; Morgans *et al.*, 1996).

In addition to their role during transmitter release, the SNARE proteins SNAP-25 and Syntaxin have additional roles during development. Disruption of SNAP-25 via anti-sense oligonucleotides or botulinum toxin disrupted neurite outgrowth and synaptogenesis (Osen-Sand *et al.*, 1993; Osen-Sand *et al.*, 1996). Differential functions of SNAP-25 during neurite outgrowth and development may be mediated via two different isoforms of the protein. SNAP-25 exists as two isoforms (SNAP-25a or SNAP-25b) via differential RNA splicing. Expression of SNAP-25a is relatively constant in the brain throughout development, while SNAP-25b expression, which is virtually absent during early development, dramatically increases as synapse formation proceeds. Thus, SNAP-25a may be the "developmental" isoform involved during neurite outgrowth, while SNAP-25b may be the "synaptic" isoform (Bark *et al.*, 1995; Bark and Wilson, 1994). Further, during regeneration of CNS processes after kainate-induced insult, the expression of SNAP-25a increases, suggesting it may play a role in process outgrowth during regeneration (Boschert *et al.*, 1996). Cleavage of Syntaxin by the use of botulinum toxin also inhibited neurite outgrowth. When peptides were used to disrupt the Synaptobrevin binding site on Syntaxin, this also disrupted neurite outgrowth, suggesting that Syntaxin's role during neurite outgrowth acts through formation of a SNARE-like complex (Boschert *et al.*, 1996; Igarashi *et al.*, 1996).

A great deal of the identification and characterization of SNARE complex, and other presynaptic terminal-associated proteins has been done biochemically (Calakos and Scheller, 1996). These studies have been extremely useful in determining the mechanisms by which these proteins interact with one another, to facilitate or otherwise regulate vesicle fusion.

Genetic studies (Calakos and Scheller, 1996; Hess, 1996; Li *et al.*, 1993; Littleton *et al.*, 1998; Mehta *et al.*, 1996) have helped to define what physiological role a given protein might play. Studies which have characterized the localization of presynaptic proteins at specific types of synapses (Catsicas *et al.*, 1992; Morgans *et al.*, 1996; Staple *et al.*, 1997) also provide valuable information as to their function at the terminal. In addition, several studies have defined additional functions for presynaptic proteins during neural development (Igarashi *et al.*, 1996; Osen-Sand *et al.*, 1993; Osen-Sand *et al.*, 1996). The cellular and synaptic organization of the vertebrate retina provides an excellent model to examine the spatial distribution of presynaptic proteins (Bachman and Balkema, 1993; Catsicas *et al.*, 1992; Grabs *et al.*, 1996; Igarashi *et al.*, 1996; Kapfhammer *et al.*, 1994; Ma *et al.*, 1995; Mandell *et al.*, 1992; Mandell *et al.*, 1990; Osen-Sand *et al.*, 1993; Osen-Sand *et al.*, 1996; West Greenlee *et al.*, 1996; West Greenlee *et al.*, 1998). Examination of the characteristic development of retinal organization in terms of the spatio-temporal distribution of presynaptic proteins may provide insight as to their developmental function.

We have characterized the spatial and temporal localization of SNARE complex as well as other presynaptic terminal proteins in the developing mammalian retina. In many mammalian species, a good deal of retinal development occurs prenatally. Thus, characterization or manipulation of early developmental events is complicated by the necessity for *in utero* procedures. We have circumvented this problem by doing a comparative study with the rat as a conventional model system, and a marsupial, the Brazilian opossum whose retinal development is largely postnatal (Keuhl-Kovarik *et al.*, 1995; West Greenlee *et al.*, 1996; West Greenlee *et al.*, 1998).

The Brazilian opossum, *Monodelphis domestica*, is a pouchless marsupial whose young are born after approximately 13.5 days of gestation. At birth the opossum pups attach to nipples on the ventral side of the mother. They remain attached until 15-20 days postnatal, at which time they are able to reattach themselves in the event of detachment. The pups are

weaned around 60 days postnatal, and are reproductively mature at six months (Kuehl-Kovarik *et al.*, 1995). They have a very protracted period of postnatal neural genesis and differentiation which make them particularly useful for studies of the developing nervous system (Guillery and Taylor, 1993; Kuehl-Kovarik *et al.*, 1993; Swanson *et al.*, 1996; West Greenlee *et al.*, 1998; West Greenlee *et al.*, 1996).

The vast majority of visual system development in *Monodelphis* is postnatal (West Greenlee *et al.*, 1996). At birth only the very first retinal ganglion cells have begun to differentiate. They have elaborated axons which are just reaching the optic chiasm, and do not reach their target structure in the brain until 5 days postnatal (5PN) (Axlund *et al.*, 1992). Eye opening in this animal occurs at approximately 35 days postnatal. However, further refinement of ganglion cell projections to the LGN continues until at least 45PN (West Greenlee and Sakaguchi, 1998). The protracted period of postnatal development allows for greater resolution of biochemical, morphological and physiological changes that accompany retinal differentiation. For this reason, marsupials, like the Brazilian opossum, have been utilized in studies of the developing visual system (Cavalcante, 1985; Dunlop *et al.*, 1996; Dunlop *et al.*, 1997; MacLaren and Taylor, 1995).

We have observed differences in the spatial and temporal localization of various presynaptic proteins in the developing mammalian retina. In addition, we have characterized transient high levels of the SNARE protein SNAP-25, in a specific sub-class of amacrine cell, the cholinergic amacrine cell, during retinal differentiation. Dynamic differences in the localization of these proteins, suggests they may play crucial roles during retinal development.

### **Dissertation Organization**

This dissertation is composed of five sections. The first section is a general introduction, which includes a literature review. Section two was published in *Developmental Brain Research* (West Greenlee *et al.*, 1996). The third section was published in *Journal of*

Comparative Neurology (West Greenlee *et al.*, 1998). The fourth section is a manuscript in preparation, which will be submitted for publication. The fifth section is a general discussion of the results presented in chapters two, three and four.

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**POSTNATAL DEVELOPMENT AND THE DIFFERENTIAL EXPRESSION OF  
PRESYNAPTIC TERMINAL-ASSOCIATED PROTEINS IN THE  
DEVELOPING RETINA OF THE  
BRAZILIAN OPOSSUM, *MONODELPHIS DOMESTICA***

A paper published in Developmental Brain Research<sup>1</sup>

M. H. West Greenlee <sup>2,3</sup>, J. J. Swanson, J. J. Simon,  
J. K. Elmquist, C. D. Jacobson, and D. S. Sakaguchi <sup>2,4</sup>

**Abstract**

In the present study we have characterized the postnatal (PN) development of the retina in the Brazilian opossum, *Monodelphis domestica*. *Monodelphis*, a small, pouchless marsupial undergoes a protracted period of postnatal development. Using bromodeoxyuridine immunohistochemistry, we have investigated postnatal neurogenesis of the retina. In addition, we have examined the differentiation of the retina by using antibodies directed against the presynaptic terminal-associated proteins Synaptotagmin, Rab3A, Synaptophysin and Synaptosomal-Associated Protein-25 (SNAP-25), and have characterized their spatial and temporal distribution during postnatal development. This study is the first systematic

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<sup>2</sup>Graduate student and Associate Professor, respectively, Department of Zoology and Genetics & Neuroscience Program, Iowa State University.

<sup>3</sup>Primary researcher and author

<sup>4</sup>Author for correspondence



comparison of the developmental expression of multiple presynaptic terminal-associated proteins in relation to retinal neurogenesis and differentiation. At birth (1PN), the *Monodelphis* retina was relatively undifferentiated morphologically and birthdating analysis revealed mitotically active cells throughout the retina. The 8PN retina was organized into two cellular layers: an outer region of mitotically active neuroepithelial cells and an inner region of postmitotic cells. The inner plexiform layer formed between 5PN and 10PN, and exhibited unique patterns of immunoreactivity with the antibodies used in this analysis. By 25PN the retina was well laminated, and Synaptotagmin-, Rab3A-, Synaptophysin- and SNAP-25-like immunoreactivities (-IR), exhibited distinct and specific patterns within the plexiform layers, although they had not yet achieved their mature, adult patterns. These results indicate that each of these proteins exhibit developmentally regulated changes in their cellular localization, and therefore may play important roles during morphogenesis and synaptogenesis of the vertebrate retina.

### Introduction

The development of the nervous system is characterized by a complex series of ordered events which include cell proliferation, migration, differentiation, process outgrowth, synaptogenesis and cell death. During synaptogenesis, axonal growth cones, are transformed into presynaptic nerve terminals upon reaching their targets. As a part of this process, the neuron must synthesize a unique set of proteins which will become integral components of the functional synapse. The molecular mechanisms involved in synaptogenesis and the proper function of presynaptic terminals require numerous interactions of unique proteins with components of the cytoskeleton and organelles localized to the nerve terminal [38]. Synaptotagmin (p65), Rab3A, Synaptophysin (p38) and Synaptosomal-Associated Protein-25 (SNAP-25) are identified presynaptic terminal proteins, each with unique functions. The characterization of their spatial and temporal distribution during synaptogenesis, and their

relationship to neurogenesis, will ultimately enhance our understanding of the complex events critical during the development of the mammalian CNS. In this study, we have used antibodies directed against these presynaptic terminal-associated proteins, as markers of synaptogenesis and have examined the development of the functional organization of the mammalian retina.

Synaptotagmin is a 65 kDa protein found in abundance in synaptic vesicles [9, 20, 64]. It is a membrane spanning protein which may act as a calcium receptor triggering neurotransmitter release [16]. Recent studies using knockouts in both mice and fruit flies demonstrated the necessity for Synaptotagmin in  $\text{Ca}^{++}$  dependent neurotransmitter release [8, 28]. A previous study examining the mature monkey retina detected Synaptotagmin in both the ribbon synapses of photoreceptors and bipolar cells, as well as in conventional synapses of amacrine cells [47].

Rab3A [24] belongs to a family of small (20-25 kDa) vesicular membrane bound GTP binding proteins. Studies with knockout mice have shown that Rab3A accelerates the efficiency of transmitter release, although it was not necessary for the release process [27]. In the mature retina, EM analysis reveals that Rab3A is present in conventional synapses like those found in amacrine cells, and non-vesicular synapses of horizontal cells, but is absent from ribbon synapses of bipolar cells and photoreceptors [29].

Synaptophysin is a 38 kDa integral membrane protein present on synaptic vesicles [43]. The relationship between the expression of Synaptophysin-like immunoreactivity (-IR) and the time course of cerebral development in the mouse suggest its expression is correlated with synaptogenesis [46]. It has also been used as a marker of a differentiated synapse [62], however, other studies suggest this protein is present in growth cones before complete morphological synapses are formed [50].

SNAP-25 [12,63] is a 25 kDa SNAP receptor (SNARE) protein associated with the presynaptic plasma membrane [5]. The expression of SNAP-25 significantly increases during synapse formation, although detectable levels are present before the onset of synaptogenesis

[62]. In the nerve terminal, SNAP-25 functions at one or more stages of vesicle docking and fusion. SNAP-25 may have important roles during development in addition to its role during neurotransmitter release [62]. Elongation of processes from neonatal rat cortical neurons and NGF-stimulated PC12 cells was prevented *in vitro*, and amacrine cell axonal elongation in the chick retina was blocked *in vivo* with the use of SNAP-25 anti-sense oligonucleotides [62]. In the mammalian retina, SNAP-25 message has been detected in amacrine cells which form conventional synapses in the inner plexiform layer (IPL); retinal ganglion cells (RGCs), which form conventional synapses in the superior colliculus; and in horizontal cells which form non-vesicular synapses in the outer plexiform layer (OPL) [13]. However, SNAP-25 is not present in ribbon synapses made by photoreceptors and bipolar cells [29].

In the retina, few studies have investigated the changing expression patterns of presynaptic terminal-related proteins during development [3, 13, 31, 44]. However, these studies did not systematically compare the distribution of several markers and their relationship of expression to differentiation. Since a great deal of neurogenesis and synaptogenesis occurs prenatally in the mammalian retina, most studies designed to examine the early development and differentiation of the retina require the use of *in utero* surgical manipulations [13]. The use of marsupials circumvents these procedures and is therefore advantageous for *in vivo* analysis of the developing visual system [1, 7, 14, 32, 33, 34, 35, 36, 37, 52, 53, 54, 55, 56, 58, 59, 76, 78]. *Monodelphis domestica*, the Brazilian opossum, is a pouchless marsupial whose young are accessible for developmental studies [26, 49, 72]. *Monodelphis* pups are born after 14 days of gestation in an extremely immature state, before neurogenesis in the CNS is complete [41, 42, 84]. For these reasons, *Monodelphis* has been used to study the development of multiple regions of the CNS including the cerebral cortex [23, 72], hypothalamus [22,23, 26, 41, 48,70, 74], brainstem [21, 48, 88], cerebellum [18], olfactory bulbs [10, 66], spinal cord [11, 60, 61, 81, 87], and the visual system [2, 29, 30, 70, 71, 83,

85, 86, 90]. Thus, the use of *Monodelphis* provides an attractive system for *in vivo* studies of the developing retina.

In the present study we have used bromodeoxyuridine (BrdU) immunohistochemistry to investigate postnatal neurogenesis of the *Monodelphis* retina. To characterize retinal development and differentiation, we have examined the spatial and temporal expression of the presynaptic terminal-associated proteins: Synaptotagmin, Rab3A, Synaptophysin and SNAP-25, and have described their distribution in relationship to periods of neurogenesis and differentiation in the mammalian retina.

## Methods and Materials

### Animals

Brazilian opossums were obtained from a colony maintained at Iowa State University. All laboratory procedures were carried out in accordance with guidelines, and had the approval of the Iowa State committee on animal care.

The animals were maintained in a constant temperature environment (26°C) on a 14:10 hour light:dark cycle with food (Reproduction Fox Chow; Milk Specialties Products, Madison, WI) and water *ad libitum*. The Brazilian opossum has a gestation period of approximately 13.5 days [49, 57]. Date of birth was designated as postnatal day 1 (1PN). Pups were weaned from the mother at 60PN.

### Tissue Preparation

We have examined retinal development in opossum pups at postnatal days 1, 5, 10, 15, and 25, as well as in adult opossums (1.5-2 years). Pups between 1 and 15PN were anesthetized by hypothermia. The animals were then decapitated and their heads fixed by immersion in either Zamboni's fixative (1.8% paraformaldehyde, 7.5% saturated aqueous picric acid in sodium phosphate buffer; pH 7.5), 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.5) or 1:1 (4% paraformaldehyde in 0.1 M PO<sub>4</sub> buffer: Histochoice

Tissue Fixative (Amresco)) for 48 hours. Postnatal day 25 pups were deeply anesthetized, and perfused transcardially with Zamboni's fixative. The heads were then post-fixed in Zamboni's fixative for 48 hours. Adult opossums were anesthetized with ether, perfused transcardially with Zamboni's fixative, the eyes removed and subsequently post-fixed for 48 hours. Tissue was cryoprotected in a 30% sucrose solution in 0.1 M  $\text{PO}_4$  buffer, embedded in OCT freezing medium (Miles) and sectioned coronally at 16  $\mu\text{m}$  or 20  $\mu\text{m}$  on a cryostat (American Optical or Reichart-Jung). Sections were thaw mounted onto poly-L-lysine (Sigma) coated or Superfrost (Fisher) microscope slides and stored at 4° C or -20° C until processed. At least three retinæ, from two animals of different litters were used in the analysis for each developmental time point examined.

### **Bromodeoxyuridine (BrdU) Analysis**

Procedures using BrdU to identify newly generated cells were performed as previously described by Iqbal and colleagues [41]. Postnatal day 1 and 8 opossum pups were injected subcutaneously along the dorsal midline with 20  $\mu\text{l}$  of BrdU solution (1  $\mu\text{g}/\text{ml}$ ) (Sigma) in 0.9% NaCl while still attached to their anesthetized mothers. The pups were allowed to survive for an additional two hours at which time they were anesthetized by hypothermia and prepared for immuno-histochemical analysis as described above. The tissue sections were rinsed with 50 mM potassium phosphate buffered saline (KPBS; 0.15 M NaCl, 0.034 M  $\text{K}_2\text{HPO}_4$ , 0.017 M  $\text{KH}_2\text{PO}_4$ ), and pretreated with 0.06% trypsin (Bovine type III; Sigma) and 0.06%  $\text{CaCl}_2$  in KPBS for 30 minutes at 37° C. After washing for 10 minutes with KPBS, the tissue sections were treated with 0.1 N HCl (ice cold) for 10 minutes followed by incubation in 2 N HCl at 37° C for 30 minutes. The tissue sections were then neutralized in basic KPBS (pH 8.5) and subsequently processed for routine immunohistochemistry (see below).

## **Immunohistochemistry**

The protocol utilized for immunohistochemistry was a modification of that previously described by Elmquist and colleagues [21]. Slide mounted tissue sections were rinsed in KPBS, and endogenous peroxidase activity eliminated by a 30 minute incubation in 0.3% hydrogen peroxide solution in KPBS. The sections were then incubated for two hours in blocking solution (KPBS with 1% bovine serum albumin (BSA; Sigma), 0.4% Triton X-100 (Fisher), and 1.5% normal blocking serum (NBS, horse or goat; Vector Laboratories Inc., Burlingame, CA)), and incubated overnight at room temperature (23°C) in the respective primary antibodies (see below). On the following day, tissue sections were rinsed in KPBS with 0.2% Triton X-100 and incubated in an appropriate biotinylated secondary antibody for two hours at room temperature, rinsed, and incubated in Avidin-Biotin Complex for 1 hour at room temperature (Vector Elite ABC Kit; 1:200 or 1:600). In order to visualize the antibody staining pattern, the tissue was reacted with a substrate of 0.4% 3',3' diaminobenzidine tetrahydrochloride (DAB; Sigma or Amresco), 2.5% nickel sulfate (Sigma) and 0.05% hydrogen peroxide (Fisher), in 0.1 M sodium acetate (Fisher). The reaction was terminated in successive rinses of 0.9% NaCl solution. Some sections were counterstained in 20% neutral red (Fisher) in 0.2 M sodium acetate (pH 5.2). Sections were then dehydrated through a graded ethanol series, cleared with xylene or Americlear (Baxter), and coverslipped with Permount (Fisher) or Accumount (Baxter). Negative controls were run in parallel during the immunohistological processing by the omission of the primary or secondary antibodies. No antibody staining was observed in the controls.

## **Antibodies**

Synaptotagmin [9, 64] and Rab3A [24] antibodies were generously provided by Dr. Reinhard Jahn of the Howard Hughes Medical Institute, Yale University School of Medicine. Synaptophysin antibody [43] was generously provided by Dr. Andrew Czernik of the

Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University.

Synaptosomal-Associated Protein-25 (SNAP-25) antibody [12, 63] was generously provided by Dr. M. C. Wilson of the Department of Neuropharmacology, Scripps Research Institute. These primary antibodies were diluted at 1:2000. Anti-BrdU antisera (Mouse monoclonal, Dako) was diluted 1:200. All primary antibodies were diluted in KPBS with 1% BSA, 0.4% Triton X-100, and 1% NBS. Biotinylated horse anti-mouse, and goat anti-rabbit secondary antibodies (Vector Laboratories Inc.) were diluted at 1:600 for presynaptic terminal analysis, and at 1:200 for BrdU analysis. All secondary antibodies were diluted in KPBS with 1% BSA, 0.02% Triton X-100, and 1% NBS. The specificity of Synaptotagmin, Synaptophysin, and SNAP-25 have been previously examined in immunoblot analysis [84]. These antibodies detected specific bands with apparent molecular weights of 65 kDa for Synaptotagmin, 38 kDa for Synaptophysin, and 25 kDa for SNAP-25.

### **Analysis of Tissue Sections**

Tissue sections were examined with a Nikon Microphot FXA photomicroscope and photographed using Kodak TMAX 400 black and white print film or Fujicolor 400 color print film. Images were also captured using a Kodak Megaplug Camera (Model 1.4) connected to a Perceptics MegaGrabber framegrabber in a Macintosh 8100/80 computer (Apple Computer, Cupertino, CA) using NIH Image 1.55 software (Wayne Rasband, National Institutes of Health, Bethesda, MD). Figures were prepared using Adobe Photoshop Version 3.0 and Aldus Freehand Version 4.0 for Macintosh. Outputs were generated on a Tectronix phaser continuous tone color printer.

## Results

### Postnatal neurogenesis in the *Monodelphis* retina

At birth (1PN) the *Monodelphis* retina is relatively undifferentiated morphologically, and resembles a pseudostratified epithelium in transverse section (Fig. 1A). In order to investigate cellular genesis in the retina we have used bromodeoxyuridine (BrdU) immunohistochemistry. Analysis of BrdU-labeled tissue revealed that extensive neurogenesis was occurring at birth and continued well into the postnatal period of development. Injections of BrdU into 1PN opossum pups resulted in labeling throughout most of the retina with the exception of a region in dorso-central retina along the inside of the eyecup (Fig. 1A). This region was composed of unlabeled postmitotic cells, many of which are likely to be RGCs (Fig. 1A).

Injections of BrdU into 8PN pups resulted in extensive labeling in the outer one-half of the neural retina (Fig. 1B). At this age the retina could be subdivided into two distinct regions: an inner half, composed of BrdU-negative postmitotic cells that exhibited a rounded morphology, and an outer neuroblastic layer (NBL), composed of elongated BrdU-labeled cells, as well as unlabeled, postmitotic cells (Fig. 1B). These results confirm that neurogenesis in the *Monodelphis* retina continues well into the postnatal period.

### Developmental expression of presynaptic terminal-associated proteins

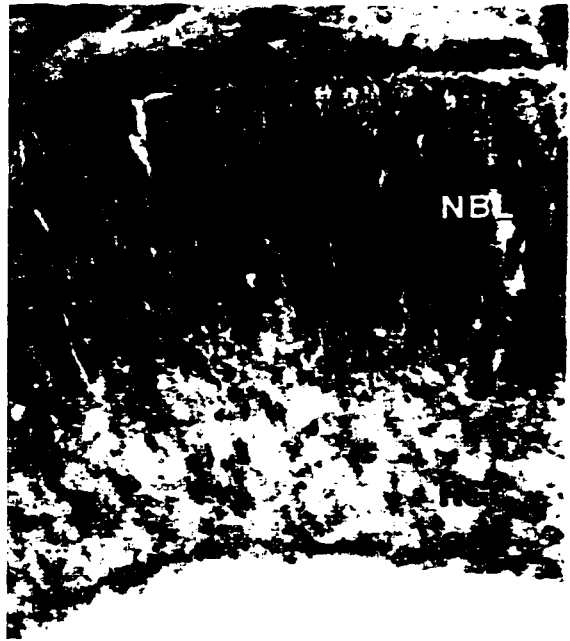
Using specific antibodies directed against Synaptotagmin, Rab3A, Synaptophysin, and SNAP-25 we have examined the spatial and temporal distribution of these presynaptic terminal-associated proteins at various postnatal ages from birth to adulthood. Analysis of the patterns of immunoreactivity revealed that each presynaptic terminal-associated protein undergoes changes in their distributions during the development of the mammalian retina.

#### 1PN

At 1PN Synaptotagmin and Rab3A-like immuno-reactivity (-IR) was observed primarily along the inner portion of central retina in presumptive RGCs and their axons in the optic fiber



**Figure 1: Birthdating analysis using bromodeoxyuridine (BrdU) immuno-histochemistry in the postnatal *Monodelphis* retina. A coronal section through the *Monodelphis* retina from a 1PN animal injected with BrdU exhibits BrdU-labeled cells throughout the retina with the exception of a region in dorso-central retina along the inner portion of the eyecup (A). In contrast, the retina of an 8PN animal injected with BrdU exhibits BrdU-labeled cells only in the outer one-half of the retina, while the inner half is composed of BrdU-negative postmitotic cells (B). Scale bars = 20  $\mu$ m. Abbreviations: NBL, neuroblastic layer; OFL, optic fiber layer; ON, optic nerve; RGC, retinal ganglion cells; RPE, retinal pigment epithelium.**



**5PN**layer (OFL) (Fig. 2 A, D). Labeled ganglion cell axons were also observed exiting the eye at the optic nerve head region. Synaptophysin and SNAP-25-IR was not observed at birth (Fig. 3 A, D).

At 5PN, the retina was organized into two cellular layers: an inner region of postmitotic cells, and an outer neuroblastic layer composed of elongated neuroepithelial cells and postmitotic cells. Immuno-reactivity for Synaptotagmin and Rab3A was still prevalent along the inner retina in the OFL and the RGC body layer with immunoreactive processes extending one-third to one-half the distance outward towards the retinal pigment epithelium (RPE) (Fig. 2B, E). Staining for Synaptophysin and SNAP-25 was first detected around 5PN. Light Synaptophysin-IR (Fig. 3B) was observed in the somata of presumptive RGCs along the inner one-third of the retina. In contrast, SNAP-25-IR (Fig. 3E) resembled the Synaptotagmin and Rab3A labeling pattern. In addition, occasional cell bodies and processes directed inward were also labeled in the outer portion of the retina with this antibody, possibly representing newly differentiating photoreceptors and their terminal endfeet (Fig. 3E).

## **10PN**

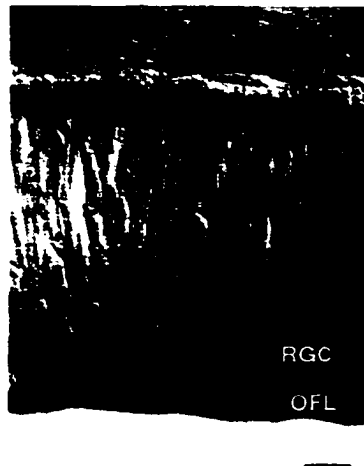
The laminar organization of the 10PN retina was clearly evident. The OFL had thickened and the RGC layer was several cell-body-diameters thick in the central portion of the retina. The nascent inner plexiform layer (IPL) was observed separating the RGC layer from the inner nuclear layer (INL); however, the outer plexiform layer (OPL) was not obvious at this period of retinal development. Synaptotagmin-IR was observed in the OFL and the IPL. Punctate immunoreactivity at the outermost portion of the retina was observed, though difficult to distinguish due to its close apposition to the RPE. Labeled cell bodies in the INL were also observed, many of which possessed darkly labeled processes extending radially outward (Fig. 2C). Rab3A-IR (Fig. 2F) was observed in the OFL and the RGC layer. Synaptophysin-IR (Fig. 3C) was observed in the outer-most portion of the neural retina (Fig. 3C and inset). The

**Figure 2: Expression of Synaptotagmin (A, B, C) and Rab3A(D, E, F) in the *Monodelphis* retina at 1PN, 5PN and 10PN. A *Monodelphis* retina at 1PN exhibits immunoreactivity for Synaptotagmin (A) and Rab3A (D) in central retina in the area of presumptive RGCs and their axons in the OFL (arrows in D) which can be observed exiting the eye as the optic nerve (ON). Immunoreactivity for Synaptotagmin at 5PN (B) remained prevalent along the inner retina, while more extensive Rab3A-like immunoreactivity(-IR) (E) often displayed somata with processes directed toward the RPE (arrows in E). Synaptotagmin-IR in the 10PN retina was observed in the newly formed IPL and the OFL (C). Rab3A-IR at 10PN was observed in the OFL and in RGC somata (F). A and D scale bars = 50  $\mu$ m; B, C, E and F scale bars = 20  $\mu$ m. Abbreviations: INL, inner nuclear layer; IPL, inner plexiform layer; NBL, neuroblastic layer; ON, optic nerve; OFL, optic fiber layer; RGC, retinal ganglion cell layer; RPE, retinal pigment epithelium.**

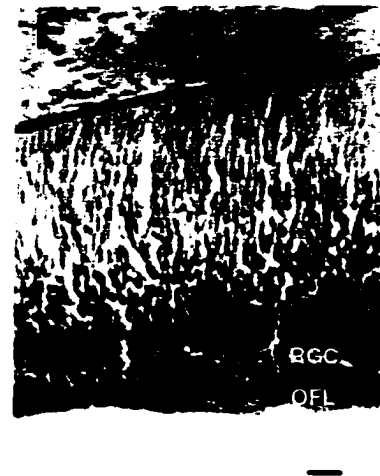
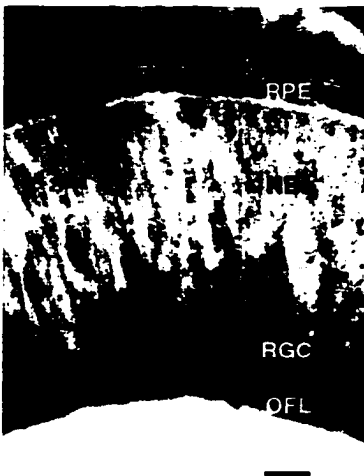
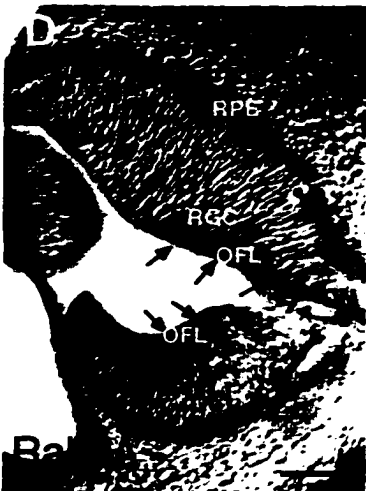
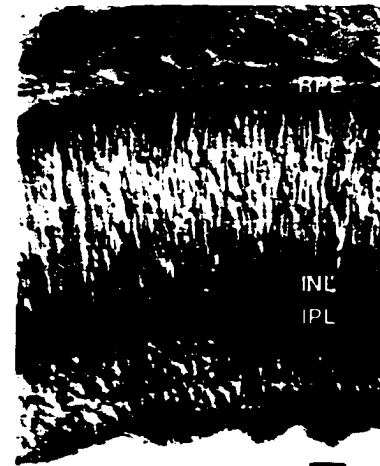
1PN



5PN



10PN



**Figure 3: Expression of Synaptophysin (A, B, C) and SNAP-25 (D, E, F) in the *Monodelphis* retina at 1PN, 5PN and 10PN. Immunoreactivity for Synaptophysin (A) and SNAP-25 (D) was not detected at 1PN. By 5PN, Synaptophysin-IR was observed in presumptive RGC somata along the inner one-third of the retina (arrows in B). SNAP-25-IR at 5PN was present within the inner one third of the retina, including the OFL (E). In addition, SNAP-25-IR was observed in the outer portion of the retina in cell bodies with processes directed inward (arrows in E). Synaptophysin-IR at 10PN was observed in the outer retina just vitreal to the RPE (inset and arrow in C). The inset in C is a higher magnification image of the region indicated by the arrow. SNAP-25-IR at 10PN was light in the fiber layers, while intense immunoreactivity was observed just vitreal to the RPE in elongated cell bodies with processes directed inward (F). Inset in C scale bar = 10  $\mu$ m; A and D scale bars = 50  $\mu$ m; B, C, E and F scale bars = 20  $\mu$ m. Abbreviations: INL, inner nuclear layer; IPL, inner plexiform layer; NBL, neuroblastic layer; ON, optic nerve; OFL, optic fiber layer; RGC, retinal ganglion cell layer; RPE, retinal pigment epithelium.**

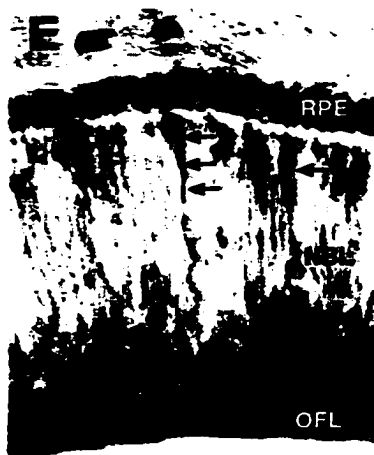
1PN



5PN



10PN



optic nerve was immunoreactive with this anti-body (data not shown), providing evidence for the presence of this protein in axons of RGCs. Relatively light SNAP-25-IR was observed in the OFL and the IPL (Fig. 3F). More extensive reactivity was observed in the outer portion of the retina, in the area of presumptive photoreceptors. Elongated cells immediately vitreal to the RPE were reactive and many also displayed thin processes directed inward (Fig. 3F).

### **15PN**

At 15PN, the RGC layer was still several cell diameters thick. Although the IPL had increased in thickness, the OPL was not yet obvious. Synaptotagmin-IR was observed very lightly in the OFL, while, more intense reactivity was observed in the IPL (Fig. 4A). Rab3A-IR was present in the OFL and IPL (Fig. 4D). Rab3A-IR was also observed in cell bodies in the RGC layer and INL adjacent to the IPL (arrows in Fig 4D). Synaptophysin-IR was observed in the IPL (Fig. 5A). Similar to the 10PN retina, Synaptophysin-IR was observed in the optic nerve (data not shown), and immunoreactivity in the outermost retina persisted, though difficult to distinguish due to its close apposition with the RPE. Immunoreactivity was also observed in somata adjacent to the IPL in the RGC layer and INL (arrows in Fig. 5A). SNAP-25-IR was present in the IPL, and in many cell bodies in the RGC layer and INL (Fig. 5D). SNAP-25-IR was also present in the outer portion of the retina, labeling processes extended inward (Fig. 5D).

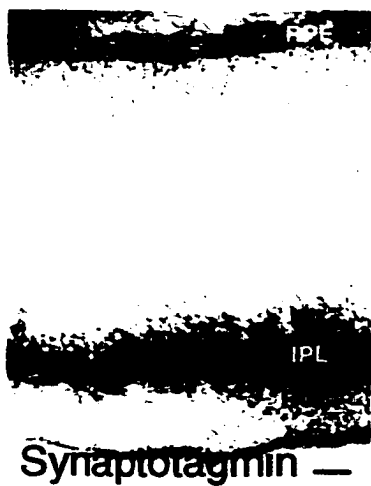
### **25PN**

By 25PN the retina exhibited all nuclear and plexiform layers, although still somewhat morphologically immature compared to the adult retina. The IPL appeared to possess the five distinct sublayers associated with the mature vertebrate retina [45,51]. Immunoreactivity for Synaptotagmin was present in the IPL while the OPL exhibited a thin band of lighter reactivity (Fig. 4B). Rab3A-IR was extensive in both plexiform layers (Fig 4E). Synaptophysin-IR

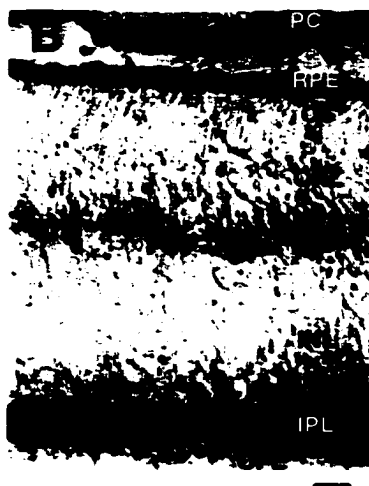


**Figure 4: Expression of Synaptotagmin (A, B, C) and Rab3A (D, E, F) in the *Monodelphis* retina at 15PN, 25PN and in the adult. Immunoreactivity at 15PN for Synaptotagmin was present in the OFL and IPL (A). Rab3A-IR was also present in the OFL and IPL. Additional Rab3A-IR was observed in cell bodies in the RGC layer and the INL adjacent to the IPL (arrows in D). In the 25PN retina, all nuclear and plexiform layers were present. Synaptotagmin-IR at 25PN displayed a uniform distribution in the IPL while the OPL exhibited a light band of immunoreactivity in the central portion of the retina (B). Rab3A-IR at 25PN was present in the OFL, IPL and in the OPL of central retina (E). In the adult retina Synaptotagmin-IR was present in the IPL and OPL (C). Rab3A-IR in the adult retina was observed in the IPL and OPL (F). Scale bars = 20  $\mu$ m. Abbreviations: INL, inner nuclear layer; IPL, inner plexiform layer; OFL, optic fiber layer; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segments; PC, pigmented choroid; RGC, retinal ganglion cell layer; RPE, retinal pigment epithelium.**

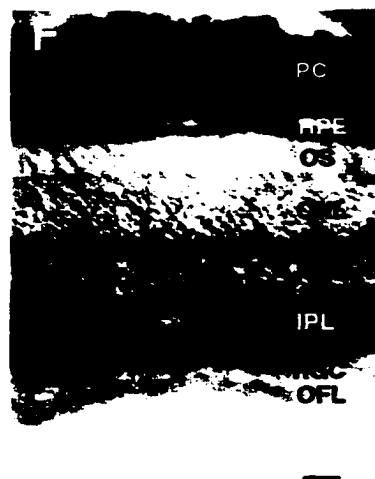
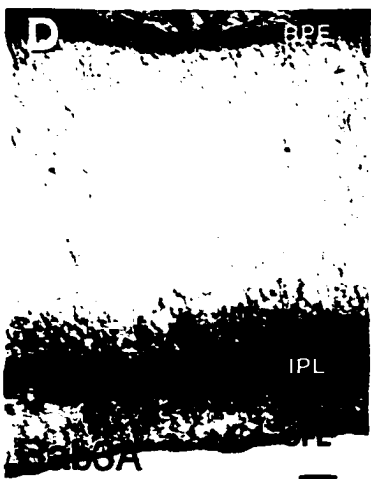
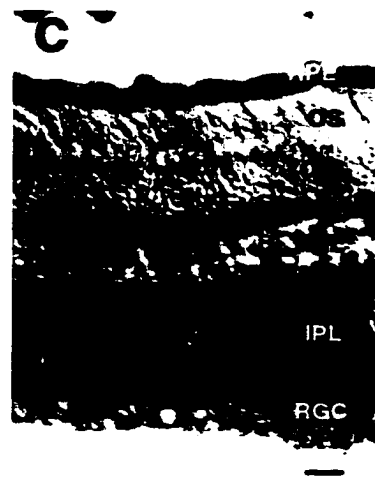
**15PN**



**25PN**

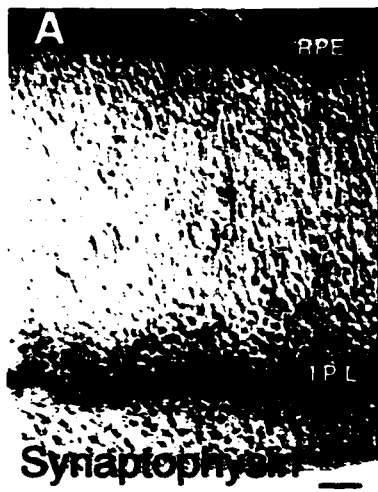


**Adult**

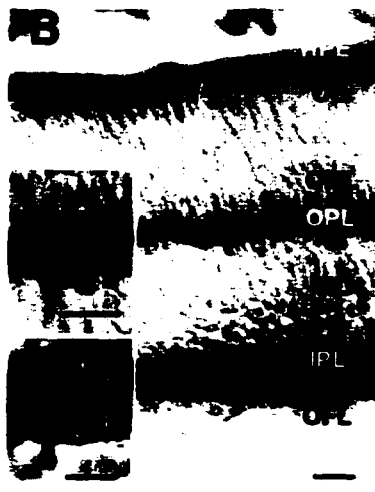


**Figure 5: Expression of Synaptophysin (A, B, C) and SNAP-25 (D, E, F) in the *Monodelphis* retina at 15PN, 25PN and in the adult. Synaptophysin-IR at 15PN was observed in the IPL (A). Lighter immunoreactivity was also observed in somata of the RGC layer and INL (arrows in A). SNAP-25-IR at 15PN exhibits a lightly reactive IPL and darkly IR cell bodies in the RGC layer and INL with reactivity often outlining the cell bodies (D). In addition, extensive immunoreactivity was observed in the outer retina, similar to that observed in the 10PN retina. At 25PN, Synaptophysin-IR in the IPL of peripheral retina demarcated three bands of darker reactivity (numbered 1, 3, and 5 in bottom inset of B), while the sublamina were not distinguishable in central retina (B). Immunoreactivity in the OPL was observed in the PR endfeet with radially directed processes observed connecting them to somata in the ONL (top inset of B). SNAP-25-IR at 25PN was observed in the OFL, IPL and the OPL (E). Immunoreactivity in the IPL consisted of two intensely labeled sublamina (numbered 2 and 4 in inset of E) separating three regions of lower reactivity (sublamina numbered 1, 3, and 5 in inset of E). Immunoreactivity was also observed in somata in the RGC layer and INL; the somata in the RGC layer were often multi-polar and extended processes directed towards the OFL (inset of E). In the adult retina, Synaptophysin-IR was observed in the OPL and IPL (C). Immunoreactivity in the IPL demarcated three darkly labeled bands with the innermost being the most intense (numbered 1, 3, and 5 in C), separated by sublamina 2 and 4 which were lightly immunoreactive. In peripheral retina SNAP-25-IR was in the OPL and the IPL (F). In central retina (F'), SNAP-25-IR was observed in the OPL, however, the reactivity within the IPL was relatively light compared to peripheral retina, and the OFL was significantly more reactive (F'). Scale bar: B top inset and E inset = 10  $\mu$ m, B bottom inset scale bar = 5  $\mu$ m, all other scale bars = 20  $\mu$ m. Abbreviations: INL, inner nuclear layer; IPL, inner plexiform layer; OFL, optic fiber layer; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segments; PC, pigmented choroid; RGC, retinal ganglion cell layer; RPE, retinal pigment epithelium. Numbers in insets correspond to sublamina of the IPL.**

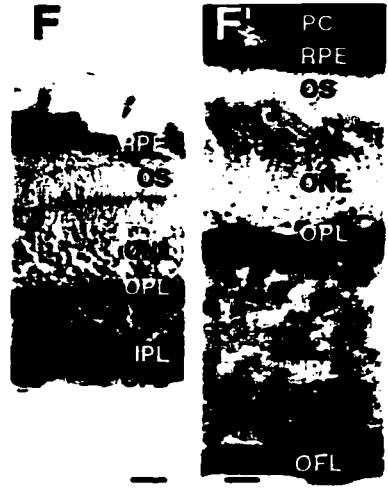
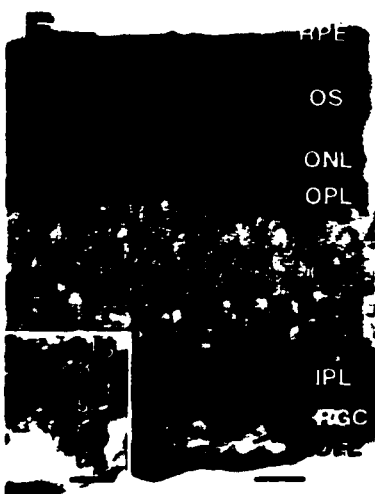
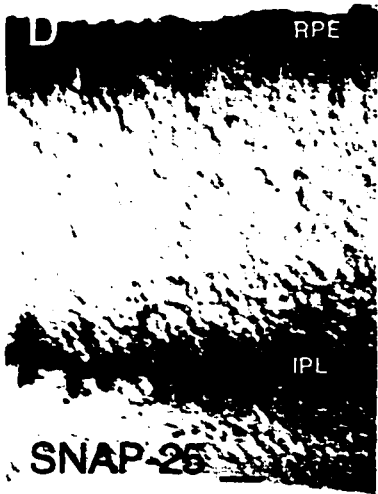
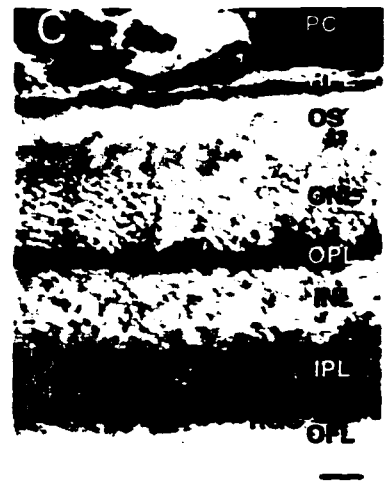
**15PN**



**25PN**



**Adult**



(Fig. 5B) was also restricted to the IPL and OPL. The reactivity in the IPL appeared to label sublayers 1, 3 and 5 more intensely, particularly in peripheral retina (Fig. 5B bottom inset), while the labeling in the OPL appeared to be present in the photoreceptor terminal endfeet (top inset in Fig. 5B). Additional immunoreactivity was observed in thin, radially oriented processes connecting the terminal endfeet to photoreceptor somata in the outer nuclear layer (ONL). SNAP-25-IR was in all layers of the 25PN retina. Immunoreactivity in the outer portion of the retina was localized to processes and somata, as was observed at earlier ages. The OPL was demarcated by a thin band of darkly labeled processes. The labeling pattern in the IPL was very distinct; two discrete bands which appeared to correspond to IPL sublayers 2 and 4 were darkly labeled while sublayers 1, 3 and 5 displayed lighter immuno-reactivity (Fig. 5E inset). SNAP-25-IR also appeared to be localized to a subset of darkly labeled cells in the RGC layer immediately adjacent to the IPL. The somata were often multipolar and occasionally displayed two processes directed towards the OFL (Fig. 5E inset). Immunoreactive cell bodies along the inner and outer portion of the INL were observed as well.

### **Adult**

In the adult retina, Synaptotagmin (Fig. 4C) and Rab3A-IR (Fig. 4F) was observed in the IPL and OPL. Light Rab3A-IR was also observed in cell bodies of the INL, particularly those adjacent to the IPL and OPL, presumably amacrine and horizontal cells, respectively. Synaptophysin-IR demarcated three sublayers within the IPL (sublayers 1, 3 and 5) (Fig. 5C) similar to those observed in the 25PN retina (Fig. 5B). However, immuno-reactivity of sublayer 5 appeared more intense than 1 or 3 (Fig. 5C). Synaptophysin-IR in the OPL appeared to be associated with photoreceptor terminal endfeet and occasional radially directed processes in a similar pattern as the 25PN retina. SNAP-25-IR (Fig. 5F, F') was uniformly present in the OPL. The IPL was darkly labeled in peripheral retina (Fig. 5F), while central

retina (Fig. 5F') displayed lighter immunoreactivity, possibly due to a differential distribution of amacrine cell densities between central and peripheral retina. SNAP-25-IR was not detected in the OFL of peripheral retina (Fig. 5F), while intensely labeled optic fibers were observed in central retina (Fig. 5F'). Occasional cell bodies in the RGC layer and INL were also immunoreactive.

### Discussion

In the present analysis we have used immunohistochemistry to examine the postnatal development and differentiation of the mammalian retina. We have used BrdU immunohistochemistry to demonstrate that the period of neurogenesis in the *Monodelphis* retina extends well into postnatal development. Antibodies directed against Synaptotagmin, Rab3A, Synaptophysin and SNAP-25 were used to systematically examine the spatial and temporal patterns of expression of these proteins during neurogenesis and differentiation of the mammalian retina. From this immunohistochemical analysis it was clear that these presynaptic terminal-associated proteins exhibited developmentally regulated changes in their cellular localization. Their expression during critical periods of neurogenesis and synaptogenesis suggests, they may be essential for the establishment of appropriate retinal connections.

#### Postnatal neurogenesis in the *Monodelphis* retina

BrdU, a thymidine analog, has become a useful alternative to tritiated thymidine for birthdating analyses in the vertebrate CNS [41, 59]. BrdU becomes incorporated into the DNA of cells actively engaged in DNA synthesis and can be detected using standard immunohistochemical procedures. BrdU injections at 1PN revealed that much of the retina, especially peripheral regions, were composed of darkly labeled BrdU-positive neuroepithelial cells. In contrast, the central retina was BrdU-negative consistent with the central-to-peripheral pattern of retinal histogenesis seen in other vertebrate retinas [67]. Extensive neurogenesis was still observed at 8PN, though BrdU-labeling was restricted to the outer half of the retina.

Thus, similar to other marsupials, and in contrast to eutherians, it appears that the majority of neurogenesis occurs post-natally in the *Monodelphis* retina.

### **Developmental distribution of Synaptotagmin, Rab3A, Synaptophysin and Synaptosomal-Associated Protein-25 in the *Monodelphis* retina**

At 1PN, the *Monodelphis* retina was relatively undifferentiated with mitotically active neuroepithelial cells occupying the majority of the retina. However, postmitotic neurons that have elaborated axons were present within the central-most region of the retina. Previous results from our laboratory have revealed numerous neurofilament-IR axons and somata within this region, providing evidence for the early genesis of RGCs [83]. The pioneering axons of these first RGCs have grown through the optic chiasm and proceeded a short distance into the contralateral optic tract [2]. Although Synaptotagmin and Rab3A-IR was observed in the central retina, the lack of expression of Synaptophysin or SNAP-25 suggests that synaptogenesis was not yet occurring [12, 46].

At 5PN, the *Monodelphis* retina was organized into two cellular layers, an inner postmitotic layer, and an outer neuroblastic layer. By this age, the first RGC axons have reached the contralateral superior colliculus, and will soon begin elaborating terminal arborizations [2]. While both Synaptotagmin and Rab3A-IR was present in the central retina, Rab3A-IR was much more extensive and labeled cells with radially oriented processes, likely representing newly generated INL cells or RGCs. The expression of Synaptophysin and SNAP-25 suggests the possible onset of synaptogenesis [12, 46] within the inner portion of the retina while neurogenesis is ongoing. Synaptophysin-IR was restricted to somata within the inner region of the retina, and evidence of axonal transport was not yet apparent. In contrast, the presence of SNAP-25-IR in the OFL suggests axonal transport of this protein may precede that of Synaptophysin.

At 10PN, the *Monodelphis* retina exhibited a rudimentary lamination pattern. The IPL was clearly observed separating the RGC somata from the outer retina, while the OPL was not yet distinguishable. By 10PN, the retinocollicular projection displays prominent contralateral and ipsilateral projections [2]. The axonal transport of all four presynaptic terminal-associated proteins was evident at this stage of development based on the presence of immunoreactivity in axonal and terminal areas. The extensive SNAP-25-IR in the outer retina provides evidence that SNAP-25 may play a role in the differentiation of different retinal cell types.

Few morphological differences were apparent between the 10PN and the 15PN retina. The RGC layer was still several somata in thickness in central retina, and although the IPL was more extensive, the OPL was still not yet distinguishable. Vimentin, an intermediate filament protein present in immature astrocytes [6, 15, 40] and in Müller glia [73] was also observed at 15PN [83]. Vimentin-IR was observed in astrocytes along the inner retina and in Müller cells displaying a radial pattern, spanning the retina from the inner to the outer limiting membrane [83]. During this same time period, glial fibrillary acidic protein (GFAP)-IR was detected in maturing astrocytes [23, 82] of the optic nerve head region (Jensen and Sakaguchi, unpublished observation). At this stage synaptotagmin and Rab3A-IR within somata was greatly reduced. Immunoreactivity for all four proteins was localized to fibers in the IPL, while Synaptotagmin, Rab3A, and Synaptophysin were likely undergoing transport in RGC axons.

At 25PN, approximately 10 days before eye opening, the basic lamination pattern of the *Monodelphis* retina was clearly evident. Synaptotagmin, Rab3A, and Synaptophysin were localized to the OPL and IPL, and their relative absence in the OFL into adulthood suggests that lower levels of these proteins were being transported to the ganglion cell terminals. In contrast, SNAP-25-IR was observed in the OFL at 25PN into adulthood, providing evidence for continuous axonal transport of this protein throughout maturation of the retina. At 25PN, SNAP-25-IR was present in a very distinctive pattern within the inner retina. Occasional



SNAP-25-IR somata located adjacent to the IPL were observed in both the inner nuclear and ganglion cell layers. Double-labeling studies have recently demonstrated the colocalization of SNAP-25 and choline acetyltransferase-IR within these somata providing evidence that these cells are cholinergic amacrine cells (West Greenlee and Sakaguchi, unpublished observations). In contrast, this distinctive pattern of SNAP-25-IR was not present in the adult retina. This dramatic change in immunoreactivity may reflect an important role of SNAP-25 during differentiation of particular amacrine cell subtypes. In addition, Osen-Sand and colleagues [62] selectively inhibited SNAP-25 expression and subsequently prevented neurite elongation in amacrine cells of the chick retina *in vivo*. Taken together, these results provide evidence that SNAP-25 may have important developmental roles in addition to its functional role during neurotransmitter release.

SNAP-25 has been shown to be associated with specific cell types in the mammalian retina. SNAP-25 message has been localized to ganglion and amacrine cells which form conventional synapses and horizontal cells which form non-vesicular synapses [13]. In contrast, ultrastructural analysis has provided evidence that SNAP-25 was absent from ribbon synapses of photoreceptors and bipolar cells [29]. While these studies were carried out in the mature retina, results from the present study show that SNAP-25-IR exhibits a more general distribution during development. We have observed SNAP-25-IR in the outer portion of the developing retina. This labeling was present from 5PN through 25PN (after the formation of the OPL) in the region of presumptive photoreceptors. Thus, this transient expression in the outer retina may reflect a role for SNAP-25 during the differentiation of the retina. Further studies examining presynaptic terminal-associated proteins during embryonic and early postnatal development may provide insight into additional functional roles for these proteins during the formation of the nervous system.

## Comparison of the timecourse of retinal development in mice, rats, and *Monodelphis*

Numerous studies have utilized rodents, especially mice and rats, as experimental models to study the development of the mammalian visual system [3, 4, 17, 39, 44, 65, 68, 75, 77, 79, 80, 89, 91]. Neurogenesis and differentiation in the retina, and the establishment of connections to central visual centers pre-dominately occurs prenatally in these species. In contrast, the Brazilian opossum is born after a gestational period of approximately 14 days, roughly equivalent to a 13 to 14 day-old rat embryo. Neurogenesis and differentiation in the retina begins just prior to birth and continues into the postnatal period of development (Jensen, Sonea, Jacobson and Sakaguchi, unpublished observations). These attributes make *Monodelphis* an exceptional experimental system to study visual system development.

The first postmitotic neurons of the developing retina are the retinal ganglion cells, which appear around embryonic day 11 (E11) [19] in the mouse, E12 in the rat [69] and approximately 1-2 days before birth in *Monodelphis* (Jensen, Sonea, Jacobson and Sakaguchi, unpublished observation). As the retina continues to differentiate, the morphological appearance of the IPL marks the initial stage of retinal lamination. In the mouse, the IPL is present around 3PN [25]; and in the rat, the IPL is observed around 2PN [44]. However, in *Monodelphis*, the IPL was first identified at approximately 8PN (West Greenlee and Sakaguchi, unpublished observation).

The morphological appearance of the OPL is a later occurring event in retinal histogenesis for all three species. In the mouse, the OPL was present at approximately 5PN, at which time Synaptophysin-IR exhibited its mature pattern of expression [3, 13]. The OPL in the rat retina was present and immunoreactive with Synaptophysin [44] and SNAP-25 [13] antibodies at 5PN. These results are consistent with the presence of synapses in the OPL of the 5PN rat retina as were previously observed using electron microscopy [89]. In the *Monodelphis* retina, the OPL becomes evident between 20 and 25PN (West Greenlee and Sakaguchi, unpublished

observation). Comparative analysis of the relatively protracted timecourse of development in the *Monodelphis* retina will ultimately provide important information for studies of the vertebrate visual system.

In summary, we have shown that the retina of the Brazilian opossum, *Monodelphis domestica*, exhibits an extensive period of postnatal neurogenesis and differentiation. Using immunohistochemical procedures, we have characterized the spatial and temporal distribution of Synaptotagmin, Rab3A, Synaptophysin and SNAP-25 during early postnatal development. The time dependent expression of these proteins indicates that they may be essential in retinal morphogenesis and synaptogenesis. Since a great deal of neurogenesis and differentiation of the retina occurs postnatally in *Monodelphis*, this species provides an excellent system for future *in vitro* and *in vivo* experimental manipulations for the analysis of the development of the mammalian visual system.

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**TRANSIENT, HIGH LEVELS OF SNAP-25 EXPRESSION IN  
CHOLINERGIC AMACRINE CELLS DURING POSTNATAL DEVELOPMENT  
OF THE MAMMALIAN RETINA**

A paper published in the Journal of Comparative Neurology<sup>1</sup>

M.H. West Greenlee<sup>2,3</sup>, S.K. Finley M.C. Wilson, C.D. Jacobson  
and D.S. Sakaguchi <sup>2,4</sup>.

**Abstract**

In the present study, we have examined the development of cholinergic amacrine cells in the retina of the Brazilian opossum, *Monodelphis domestica*. An antibody directed against choline acetyltransferase (ChAT) revealed that ChAT-like immunoreactivity (ChAT-IR) was first observed at 15 days postnatal (15PN). By 25PN, ChAT-IR identified two matching populations of amacrine cells in the inner nuclear and ganglion cell layers. Bromodeoxyuridine birthdating analysis coupled with immunolabeling with the anti-ChAT antibody revealed that the cholinergic amacrine cells are born postnatally, between 2 and 15 days postnatal. In addition, we have examined the differentiation of the cholinergic amacrine cells by using an antibody directed against a presynaptic terminal-associated protein, SNAP-25. Double-labeling analysis revealed that relatively high levels of SNAP-25-IR were selectively present in cholinergic amacrine cells prior to eye opening. However, in the mature retina, high levels of

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<sup>2</sup> Graduate student and Associate Professor, respectively. Department of Zoology and Genetics, Neuroscience Program, Iowa State University.

<sup>3</sup> Primary researcher and author

<sup>4</sup> Author for correspondence

SNAP-25-IR were no longer observed in the ChAT-immunoreactive amacrine cells. These results reveal a distinct period in development, prior to eye opening, when high levels of SNAP-25-IR are selectively expressed in cholinergic amacrine cells. The specificity and time course of the high levels of SNAP-25 in cholinergic amacrine cells may be critical in mediating the transient properties of these cells during visual system development.

### **Introduction**

Cholinergic amacrine cells have been identified in all mammalian retinas thus far examined (Masland et al., 1984b; Marc, 1986; Wassle et al., 1987), and are therefore likely to play important, conserved functional roles from species to species. Cholinergic amacrine cells, also known as starburst amacrine cells due to their distinctive morphology, consist of two mirror-symmetric subpopulations with their somata located in the inner nuclear layer (INL) and ganglion cell layer (GCL), and their processes ramifying principally in sublamina 2 and 4 of the inner plexiform layer (IPL), respectively (Famiglietti, 1983; Karten and Brecha, 1983; Marc, 1986). In the mature retina, cholinergic amacrine cells may be involved in the production of directional selectivity and may also have an important role in regulating the responsiveness of the ganglion cells (Ariel and Daw, 1982; Masland et al., 1984a). In addition to their function in the mature retina, cholinergic amacrine cells appear to have a distinct developmental role. Feller and colleagues (1996) have recently demonstrated the requirement of cholinergic synaptic transmission in the propagation of spontaneous retinal waves. Since starburst amacrine cells are the only cholinergic neurons in the developing mammalian retina, they appear to be the critical cellular element required for wave production (Feller et al., 1996). The spontaneous, patterned activity, generated before visual stimulation, is likely to play an essential role during the development and differentiation of synaptic connectivity within the visual pathway (reviewed in Goodman and Shatz, 1993; Shatz, 1996)



This period of spontaneous, correlated activity prior to eye opening corresponds to an exceptionally dynamic epoch in visual system development involving process outgrowth and synaptogenesis. In the developing mammalian retina, the expression of specific presynaptic terminal-associated proteins appears to be developmentally regulated (Catsicas et al., 1992; West Greenlee et al., 1996; Alexiades and Cepko, 1997) and therefore may be critical for synaptogenesis. In particular, synaptosomal-associated protein of 25 kDa (SNAP-25), is predominantly associated with neurons, and has multiple functions including neurotransmitter release (reviewed in Bark and Wilson, 1994; Sudhof, 1995; Calakos and Scheller, 1996), neurite outgrowth, differentiation, and synaptogenesis (Osen-Sand et al., 1993; Catsicas et al., 1994; Osen-Sand et al., 1996). Thus, the regulated spatial and temporal expression of SNAP-25 in the developing retina may play an integral role in the establishment of appropriate retinal circuitry.

We have examined the genesis and differentiation of cholinergic amacrine cells in relation to the differential expression of SNAP-25-like-immunoreactivity (IR) in the developing retina of the Brazilian opossum, *Monodelphis domestica*. From the onset of detectable ChAT-IR at 15 days postnatal (15PN), these cells displayed relatively high levels of SNAP-25-IR in their soma and processes relative to the SNAP-25-IR in surrounding retinal tissue. This relatively high level of SNAP-25 expression was transient, peaked around 25PN, and decreased to a lower level by the time of eye opening; around 35PN. This is the first study to report the transient and specific localization of relatively high levels of SNAP-25-IR within cholinergic amacrine cells during postnatal development prior to eye opening. The specific spatial and temporal localization within ChAT-immunoreactive amacrine cells suggests that this regulated expression of SNAP-25 may be critical for developmental plasticity in the mammalian visual system.

## **Methods and Materials**

### **Animals**

Brazilian opossums were obtained from a colony maintained at Iowa State University. The animals used to maintain the breeding colony were obtained from the Southwest Foundation for Research and Education (San Antonio, TX). Laboratory procedures were carried out in accordance with guidelines, and had the approval of the Iowa State University Committee on Animal Care. The animals were maintained in a constant-temperature environment (26°C) on a 14:10 hour light:dark cycle with food (Reproduction Fox Chow; Milk Specialties Products, Madison, WI) and water 'ad libitum'. The Brazilian opossum has a gestation period of approximately 14.5 days. The date of birth was designated as postnatal day 1 (1PN) and eye opening occurs at approximately 35PN. Pups are weaned from the mother at 60PN (Kuehl-Kovarik et al., 1995).

### **Tissue Preparation**

We have examined retinal development in opossum pups at postnatal days 1, 5, 10, 15, 20, 25, 30 and 35, as well as in adult opossums (1.5-2 years). Pups between 1 and 15PN were anesthetized by hypothermia. The animals were decapitated and their heads were fixed by immersion in 4% paraformaldehyde in 0.1M PO<sub>4</sub> buffer (pH 7.5) for 48 hours. Postnatal day 20, 25, 30 and 35 pups were deeply anesthetized with ether, and perfused transcardially with 4% paraformaldehyde. The heads were then post-fixed for 48 hours. Adult opossums were anesthetized, perfused transcardially with 4% paraformaldehyde, enucleated, and eyes were subsequently post-fixed for 48 hours. Tissue was cryoprotected in a 30% sucrose solution in 0.1M PO<sub>4</sub> buffer (pH 7.4), embedded in OCT freezing medium (Baxter Scientific, McGaw Park, IL) and sectioned coronally at 20 µm on a cryostat. Sections were thaw-mounted onto Superfrost microscope slides (Fisher Scientific, Pittsburgh, PA) and stored at -20° C until processed.

## **Immunohistochemistry**

Identification of cholinergic neurons in the *Monodelphis* retina was carried out by using immunohistochemical procedures with an antibody directed against choline acetyltransferase (ChAT), the synthetic enzyme for acetylcholine (Tucek, 1976). Previous studies have demonstrated that ChAT-immunoreactive neurons in the mammalian retina have the same morphological characteristics as the cholinergic, starburst amacrine cells (Famiglietti, 1983; Voigt, 1986). Starburst amacrine cells are the only cholinergic cells that have been described in the mammalian retina, and thus, ChAT-like immunoreactivity (ChAT-IR) and its characteristic staining pattern have been used as a marker for starburst amacrine cells (Hutchins and Hollyfield, 1987b; Dann, 1989; Feller et al., 1996)

## **Bromodeoxyuridine (BrdU) Birthdating Analysis of Cholinergic Amacrine Cells**

Procedures using BrdU to identify newly generated cells were performed as previously described by Iqbal and colleagues (1995). Female opossums with pups at various postnatal ages (1, 2, 3, 4, 5, 10, 12, 15, 20, and 25PN) were anesthetized with 2% isoflurane. The opossum pups were then injected subcutaneously along the dorsal midline with 20  $\mu$ l of BrdU solution (1  $\mu$ g/ml; Sigma Chemical, St. Louis, MO) in 0.15M NaCl while still attached to their anesthetized mothers. The pups were allowed to survive until 30 or 60 PN, at which time they were anesthetized and prepared for immunohistochemical analysis as described above. The tissue sections were rinsed with 0.5M potassium phosphate-buffered saline (KPBS; 0.15M NaCl, 0.034M  $K_2HPO_4$ , 0.017M  $KH_2PO_4$ ; pH 7.4), and pretreated with 0.06% trypsin (Bovine type III; Sigma) and  $5.4 \times 10^{-3}$ M  $CaCl_2$  in KPBS for 30 minutes at 37° C. After washing for 10 minutes with KPBS, the tissue sections were treated with 0.1N HCl (ice cold) for 10 minutes followed by incubation in 2N HCl at 37° C for 30 minutes. The tissue sections

were then neutralized in basic 0.5M KPBS (pH 8.5) and subsequently processed for routine immunohistochemistry.

Slide-mounted tissue sections were rinsed in KPBS, and endogenous peroxidase activity was eliminated by a 30 minute incubation in 0.3% hydrogen peroxide solution in KPBS. The sections were then incubated for 2 hours in blocking solution with normal horse serum (Vector Laboratories Inc., Burlingame, CA), and incubated overnight at room temperature (24°C) in anti-BrdU antibody (DAKO Corporation, Carpinteria, CA). On the following day, tissue sections were rinsed in KPBS with 0.2% Triton X-100 and incubated in biotinylated secondary antibody (Vector) for 2 hours at room temperature, rinsed, and incubated in horseradish peroxidase-avidin-biotin complex for 1 hour at room temperature (Vector Elite ABC Kit; 1:200). In order to visualize the BrdU-immunoreactive nuclei, the tissue was reacted with a substrate of 0.4% 3',3' diaminobenzidine tetrahydrochloride (DAB; Sigma), 0.16M nickel sulfate (Sigma) and 0.05% hydrogen peroxide (Fisher), in 0.1M sodium acetate (Fisher). The reaction was terminated in successive rinses of 0.15M NaCl solution. To determine which BrdU-immunoreactive cells were cholinergic amacrine cells, sections were processed for ChAT immunohistochemistry as described above, except following incubation in secondary antibody, tissues were incubated in ABC-complex (1:600) and nickel sulfate was omitted from the DAB solution. The resulting ChAT-IR appeared as a brown reaction product and could be easily distinguished from the dark-purple BrdU-IR. Sections were then dehydrated through a graded ethanol series, cleared with Americlear (Baxter), and coverslipped with Accumount (Baxter).

Negative controls were run in parallel during all immunohistological processing by the omission of the primary or secondary antibodies. No antibody staining was observed in the controls.

### **ChAT/SNAP-25 Double Label Immunohistochemistry**

Slide-mounted tissue sections were rinsed in 0.5M KPBS (pH 7.4), incubated for 2 hours in blocking solution (KPBS with 1% bovine serum albumin (BSA; Sigma), 0.4% Triton X-100 (Fisher), and 1.5% normal donkey serum (NDS; Jackson ImmunoResearch, West Grove, PA)), and incubated overnight at 24°C in anti-ChAT antibody (Chemicon International, Temecula, CA). On the following day, tissue sections were rinsed in KPBS with 0.2% Triton X-100 and incubated in biotinylated secondary antibody (Jackson). To visualize the antibody staining pattern, sections were then incubated in Texas-Red-conjugated avidin D (1:1500; Vector). After thorough rinsing, tissue sections were incubated in blocking solution (phosphate-buffered saline (PBS; 14 mM NaCl, 2.7mM KCL, 5.37mM Na<sub>2</sub>HPO<sub>4</sub>, 1.76mM KH<sub>2</sub>PO<sub>4</sub>; pH 7.4) with 5% goat serum, 0.4% BSA, and 0.2% Triton X-100) for 30 minutes, and incubated in diluted anti-SNAP-25 antibody at 4°C overnight. Following washes in PBS with 0.25% Triton X-100, SNAP-25-IR was visualized by incubation with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Fisher). After subsequent rinsing, slides were coverslipped with Vectashield fluorescence mounting medium (Vector).

### **Antibodies**

The anti-choline acetyltransferase (ChAT) antibody (produced in goat; Chemicon) was diluted at 1:200. Bromodeoxyuridine (BrdU) was detected by using an anti-BrdU antibody (mouse monoclonal; DAKO) and was diluted 1:200. Anti-synaptosomal-associated protein of 25 kDa (SNAP-25) rabbit antiserum was raised against a synthetic peptide corresponding to the carboxyl 12 residues of the mouse protein (Oyler et al., 1989) and was diluted at 1:200. Secondary antibodies were diluted in KPBS with 1.5% NBS, 1% BSA, 0.02% Triton X-100 (biotinylated donkey-anti-goat [1:200; Jackson], biotinylated horse anti-mouse [1:200; Vector]), or PBS with 5% goat serum, 0.4% BSA and 0.2% Triton X-100 (FITC-goat-anti-rabbit 1:200; Fisher). The specificity of the SNAP-25 antisera has been previously examined

in immunoblot analysis, with the antibody detecting a specific band with an apparent molecular weight of approximately 25 kDa (Swanson et al., 1996).

### **Analysis of Tissue Sections**

In this analysis, two to six eyes from different animals were examined at each time point. In order to determine the average number of labeled cells, three sections from each retina were examined and the data from all the retinas of a particular age were pooled. Within each retina, one section was at a position hemisecting the eye along the nasal-to-temporal axis, while the other two sections were approximately 240  $\mu\text{m}$  on each side. For the ChAT/SNAP-25 co-localization analysis, all ChAT-immunoreactive cell bodies were counted. SNAP-25-immunoreactive cell bodies in the GCL and inner one-half of the INL were counted as highly immunoreactive if the intensity of the label outlining the soma was comparable to that observed in sublamina 2 or 4 of the IPL, and therefore significantly brighter than the lower levels of immunoreactivity in the surrounding tissue. For the cholinergic amacrine cell birthdating analysis, darkly-labeled BrdU-immunoreactive, ChAT-immunoreactive and double-labeled (BrdU/ChAT-immunoreactive) cells were counted in each section within the inner nuclear layer (INL) and ganglion cell layer (GCL).

Tissue sections were examined with a Nikon Microphot FXA photomicroscope equipped with epifluorescence and photographed with Kodak Ektachrome 400 Elite color slide film or Fujicolor 400 color print film. Images were also captured by using a Kodak Megaplug Camera (Model 1.4) connected to a Perceptics MegaGrabber framegrabber in a Macintosh 8100/80AV computer (Apple Computer) with NIH Image 1.55VDM software (Wayne Rasband, National Institutes of Health, Bethesda, MD, obtained at FTP site: [zippy.nimh.nih.gov](http://zippy.nimh.nih.gov)).

Density plot profiles were prepared by using the NIH Image software. For this analysis, a profile line plot was obtained from digitized images of immunostained tissue sections in Figures 3 and 6. Each profile line plot corresponded to a thin strip of 37 x 917 pixels (5 x

124.5  $\mu\text{m}$ ; in Fig. 4) or 35 x 596 pixels (5 x 86  $\mu\text{m}$ ; in Fig. 7) marked by arrows in Figure 3 and arrowheads in Figure 6. This method of analysis provides information about relative intensities of staining. Although this approach does not provide evidence for differences in absolute levels of expression of the proteins of interest, this strategy allows a comparison of the relative levels of expression within the different layers and sublamina of the retina (Catsicas et al., 1992). Figures were prepared by using Adobe Photoshop Version 3.0 and Macromedia Freehand Version 5.5 for the Macintosh. Outputs were generated on a Tektronix Phaser continuous tone color printer.

## Results

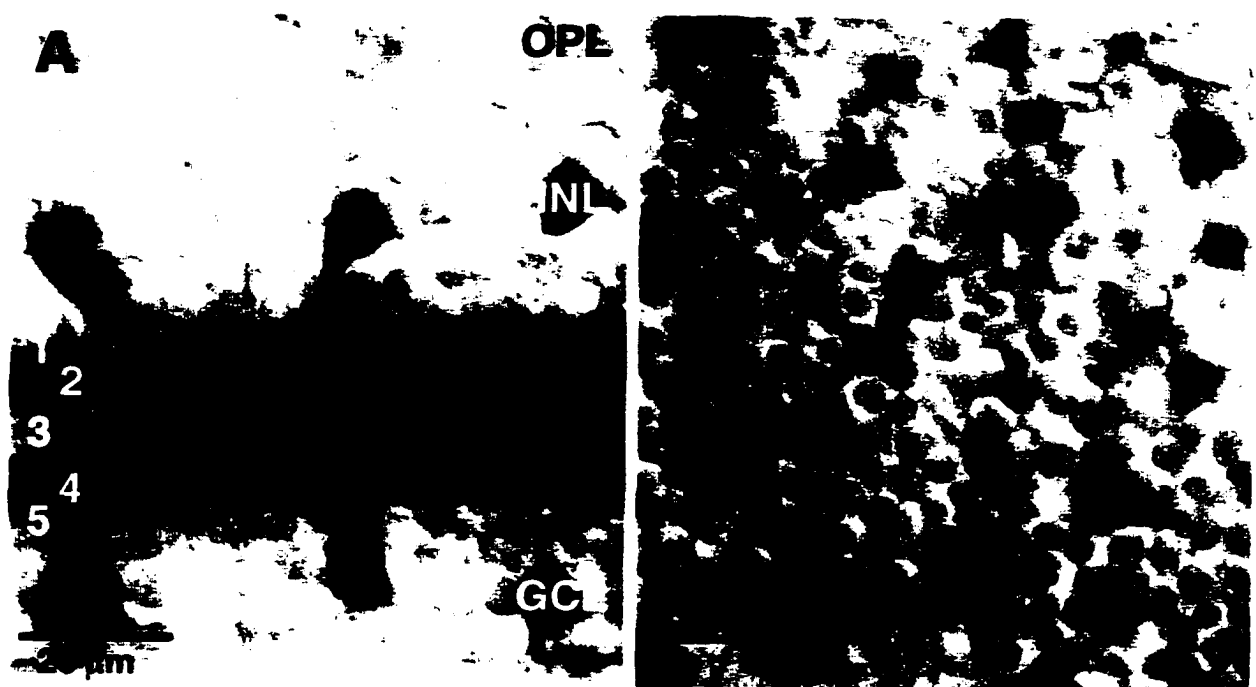
In the retina of the Brazilian opossum an antibody directed against choline acetyltransferase (ChAT) produces a distinct pattern of ChAT-IR displaying two subpopulations of cells, a conventional population in the INL and a displaced population in the GCL with processes principally in sublamina 2 and 4 of the IPL, respectively (Fig. 1A). Inspection of flat mounted retinas revealed that these two subpopulations of cholinergic amacrine cells are distributed in a relatively uniform pattern across the retina (Fig. 1B). These patterns of ChAT-IR are similar to the distribution of cholinergic, starburst amacrine cells that have been described in all mammalian retinas thus far examined (Marc, 1986; Pourcho and Osman, 1986; Voigt, 1986; Hutchins and Hollyfield, 1987a; Reese et al., 1994; Feller et al., 1996) .

### **Neurogenesis of cholinergic amacrine cells occurs during postnatal development**

To characterize neurogenesis of the cholinergic amacrine cells, we have carried out a bromodeoxyuridine (BrdU) birthdating analysis. The birth dates of the cholinergic amacrine cells were determined by double label BrdU/ChAT immunohistochemistry. Choline acetyltransferase-IR that was observed in cells which also exhibited BrdU-IR, were likely to

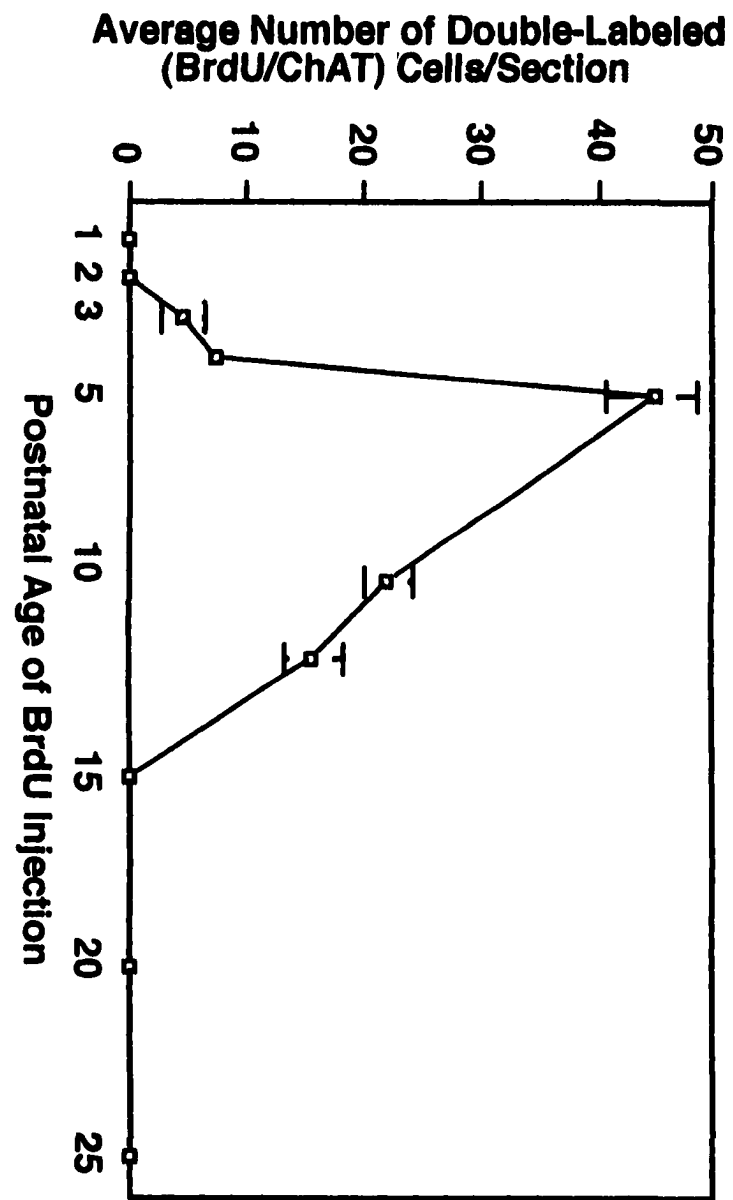
**Figure 1: Choline acetyltransferase-like immunoreactivity (ChAT-IR) in an adult retina. An antibody directed against ChAT identifies two subclasses of cholinergic amacrine cells: a conventional population in the INL and a displaced population in the GCL with processes in sublamina 2 and 4 of the IPL, respectively (A). A flat-mounted retina processed for anti-ChAT immunohistochemistry revealed that the cholinergic amacrine cells were distributed in a relatively uniform pattern across the retina (B). Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer. Numbers in A represent sublamina of the IPL. Scale bar = 20 $\mu$ m.**





**Figure 2: Cholinergic amacrine cells are born between 2 and 15PN. The vertical axis represents the average number of bromodeoxyuridine (BrdU)/ChAT double-immunoreactive cells per section. The horizontal axis represents days of postnatal development.**

**Bromodeoxyuridine injections were carried out on postnatal (PN) days 1, 2, 3, 4, 5, 10, 12, 15, 20, and 25. The first ChAT-immunoreactive amacrine cells were born around 3PN, with an approximate peak around 5PN. The number of ChAT-immunoreactive cells born on later days decreased until 15PN when no double-labeled cells were observed. Error bars represent SEM. Sample sizes for each age are as follows: 1PN (n=9 retinal sections from 3 eyes (9/3)); 2PN (n=9/3); 3PN (n=6/2); 4PN (n=6/2); 5PN(n=9/3); 10PN (n=6/2); 12PN (n=9/3); 15PN (n=6/2); 20PN (n=9/3); 25PN (n=9/3).**

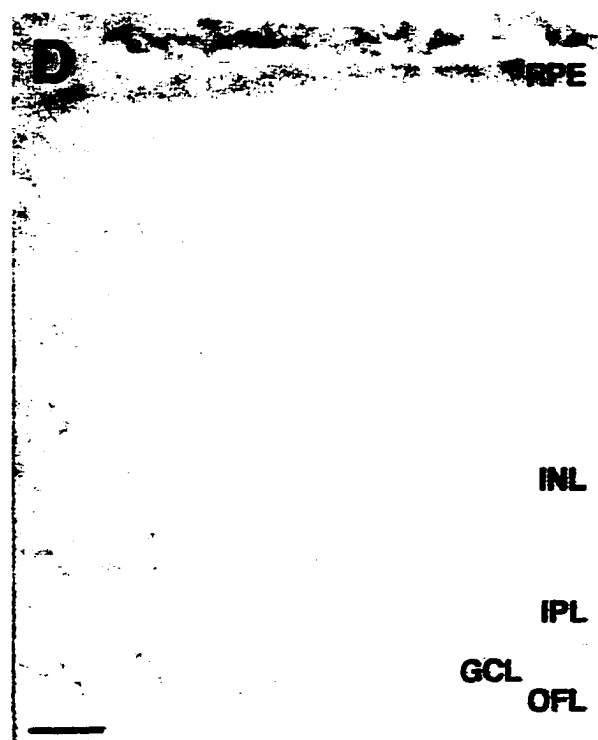
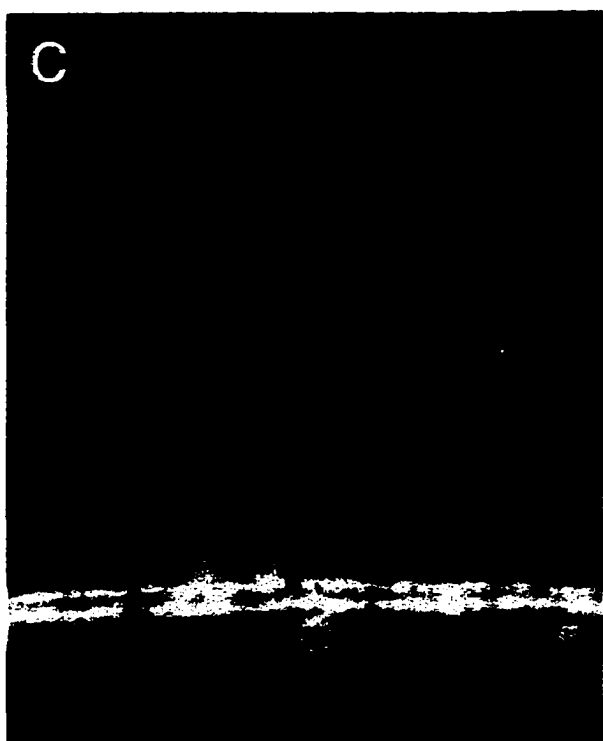
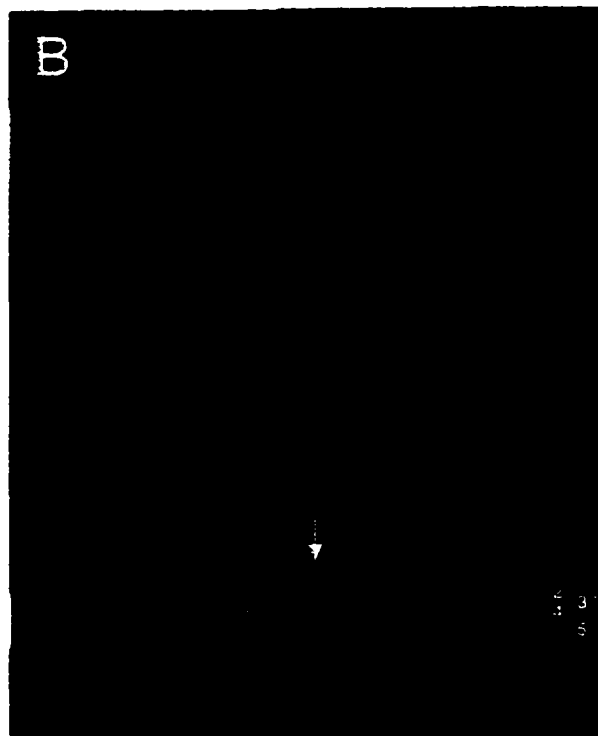
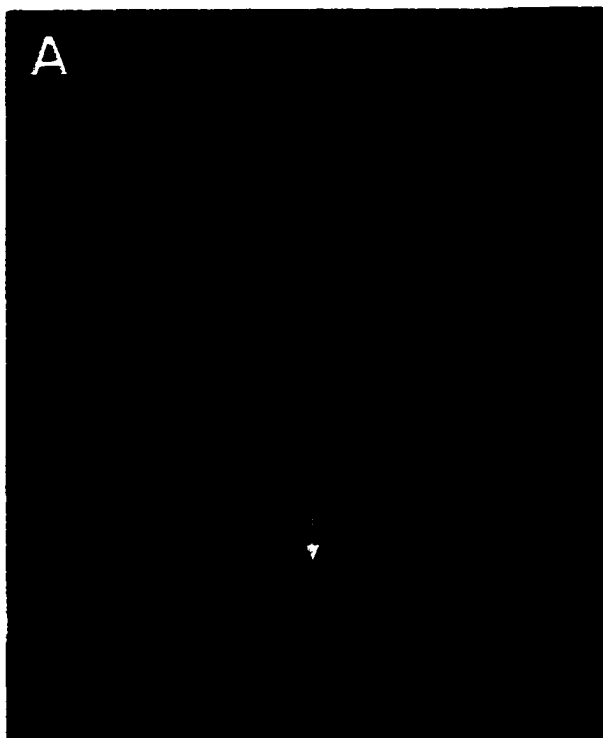


have undergone their terminal division on the day of the BrdU injection. While neurogenesis in the *Monodelphis* retina begins 1-2 days prior to birth (Finley, Jensen, Iqbal, Jacobson and Sakaguchi, unpublished observations) no double labeled cells were observed until 3PN, as illustrated in Figure 2. The genesis of ChAT-immunoreactive neurons reached a peak around 5PN (Fig. 2), after which production of ChAT-immunoreactive cells decreased until no double-labeled cells were found following BrdU-injections on day 15, 20 or 25 (Fig. 2). These results indicate that the cholinergic amacrine cells in the retina of the Brazilian opossum are born postnatally, between the ages of 2 and 15PN. Furthermore, we observe an approximate peak in the genesis of these neurons around 5PN.

### **Cholinergic amacrine cells exhibit high levels of SNAP-25 prior to eye opening**

To gain a better understanding of retinal development and differentiation, we have used immunohistochemistry to characterize the spatial and temporal distribution of presynaptic terminal-associated proteins during postnatal development (West Greenlee et al., 1996). An antiserum directed against the synaptosomal-associated protein of 25 kDa (SNAP-25) produced an intense labeling pattern remarkably similar to the distribution of the ChAT-immunoreactive amacrine cells in the 25PN retina. To investigate the possibility that these ChAT-immunoreactive amacrine cells were specifically and selectively expressing relatively high levels of SNAP-25, we have carried out a double-label immunohistochemical analysis. Figure 3A illustrates the pattern of ChAT-IR observed in a 25PN *Monodelphis* retina. Immunohistochemical analysis with the anti-SNAP-25 antiserum revealed relatively high levels of immunoreactivity outlining a subset of somata within the INL and GCL (Fig. 3B). The annular appearance of immunoreactivity provided evidence that the protein was associated with the somal membrane. Within the IPL the immunoreactivity for SNAP-25 was very distinct, exhibiting two discrete bands of intense immunoreactivity in sublamina 2 and 4. In contrast,

**Figure 3: Choline acetyltransferase-IR and high levels of SNAP-25-IR co-localize to the same cells. A, B, C: Fluorescence photomicrographs from a 25PN retina double-labeled with antibodies against ChAT (A) and SNAP-25 (B). A fluorescence double-exposure photomicrograph reveals co-localization of the two immunoreactivities in cell bodies in the INL and GCL and their processes in sublamina 2 and 4 of the IPL (C). D is a differential interference contrast image of the retinal section in A, B, and C. The arrows in A and B mark the region of retina that was analyzed by using density plot profiles illustrated in Figure 4. Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; RPE, retinal pigment epithelium. Scale bar = 50 $\mu$ m.**



relatively low levels of immunoreactivity were present in IPL sublamina 1, 3, and 5 (Fig. 3B). Double-labeling immunofluorescence revealed a selective localization of relatively high levels of SNAP-25-IR in the ChAT-immunoreactive somata (Fig 3A, B). Choline acetyltransferase- and SNAP-25-IRs were selectively co-localized in cell bodies, as well as within the processes ramifying within sublamina 2 and 4 of the IPL (Fig. 3C). Densitometric analysis clearly illustrates the relative intensities of ChAT- and SNAP-25-IR within a 25PN retina (Fig. 4A, B). Although SNAP-25-IR was also observed in the outer one-half of the retina, the relative levels of immunoreactivity were considerably less than those observed along the inner region of the retina (Fig. 3B, 4B). Within the IPL, the two peaks of highest relative intensities correspond to sublamina 2 and 4 (dashed lines in Fig. 4), while the peaks in the GCL correspond to the soma of the displaced amacrine cell (dotted lines in Fig. 4) illustrated in Figure 3A and B (arrow). This comparative densitometric analysis from the same location of retina (arrows in Fig. 3) further supports and illustrates the co-localization of the two immunoreactivities.

To further characterize the relationship of the distribution of ChAT- and SNAP-25-IRs, we examined their co-localization along the dorsal-to-ventral and nasal-to-temporal axes of the retina. Figure 5 illustrates ChAT- and SNAP-25-IRs in representative images from a section of a double-labeled 25PN retina progressing from a more dorsal (Fig. 5A, B) to a more ventral (Fig. 5K, L) retinal position. The co-localization of the two immunoreactivities was clearly evident throughout the dorsal to ventral extent of the 25PN retina (Fig. 5). In a similar fashion, analysis of sections from more nasal and temporal retinal positions also revealed co-localization of ChAT- and SNAP-25-IRs (data not shown).

### **Transient co-localization of SNAP-25 in ChAT-immunoreactive amacrine cells**

To examine the timecourse and specificity of the SNAP-25 expression in cholinergic amacrine cells, we performed a double-labeling immunohistochemical analysis on neonatal to

**Figure 4: Density plot profiles of ChAT-IR (A) and SNAP-25-IR (B) taken from the images in Fig. 3 A and B at the locations marked by the arrows. The density plot profiles permit a semi-quantitative evaluation of immunoreactivity within a retinal section (Catsicas *et al.*, 1992).**

**Choline acetyltransferase-IR was co-localized with high levels of SNAP-25-IR in the IPL (dashed lines) and in a cell body in the GCL (dotted lines). In addition, lower levels of SNAP-25-IR were present in the outer half of the retina. Baseline levels were comparable to background fluorescence observed in unlabeled and secondary antibody controls.**

**Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; RPE, retinal pigment epithelium.**



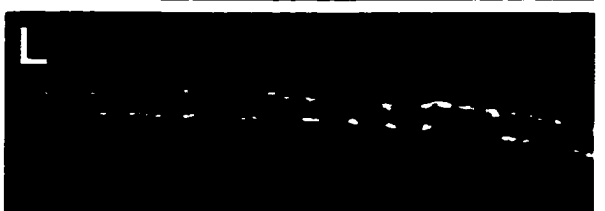
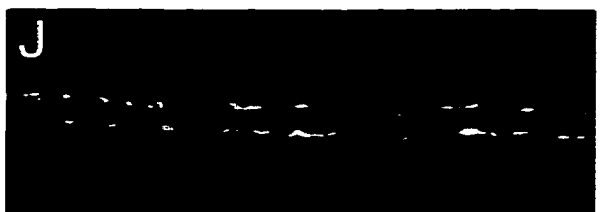
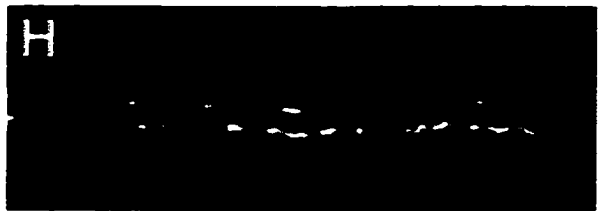
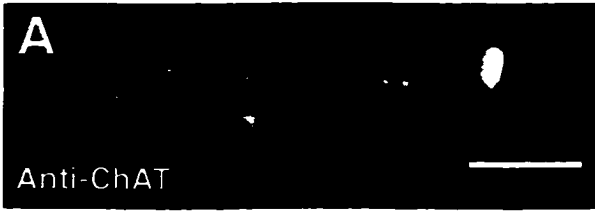


RPE Outer Retina OPL INL IPL GCL

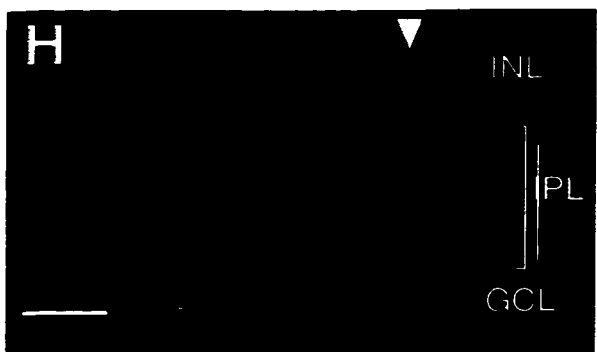
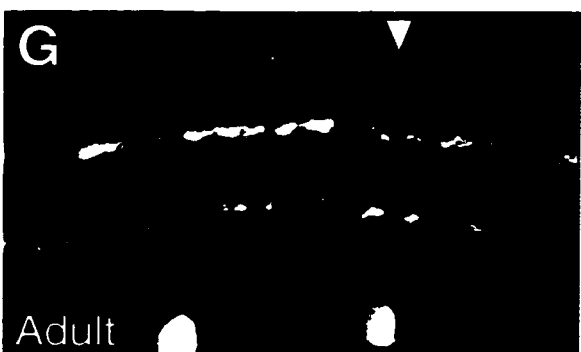
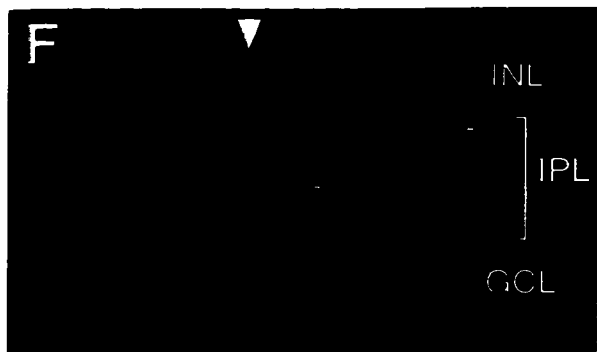
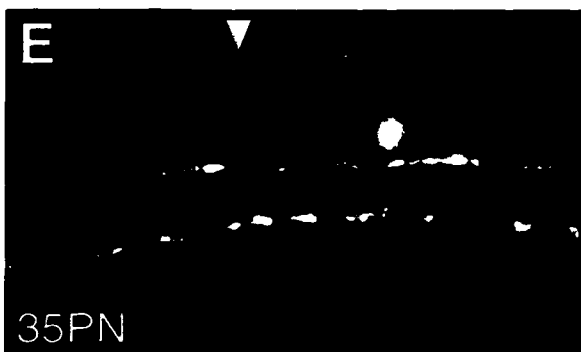
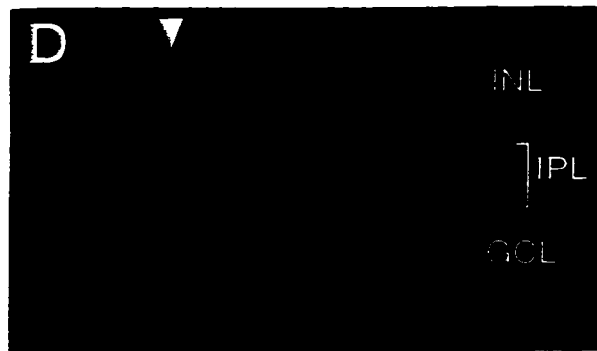
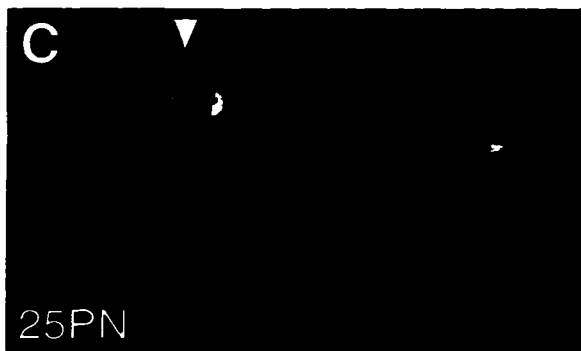
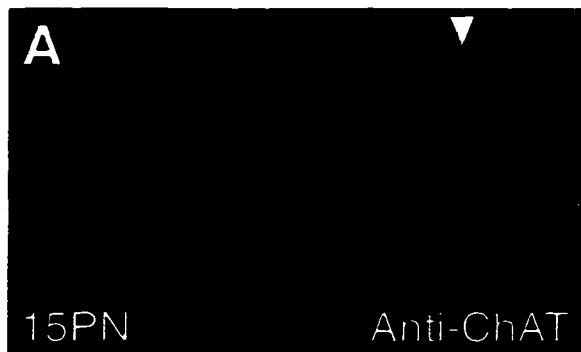
adult opossum retinal tissue. Choline acetyltransferase-IR was first observed in the 15PN *Monodelphis* retina (Fig. 6A). At this age, ChAT-IR was diffuse throughout the inner portion of the retina with a few brightly labeled cell bodies in the developing INL and GCL (Fig. 6A). Although SNAP-25-IR was detected as early as 5PN, the immunoreactivity at that age was very diffuse (West Greenlee et al., 1996). However, at 15PN, SNAP-25-IR resembled the pattern of reactivity observed with the anti-ChAT antibody (Fig. 6B). The co-localization of their immunoreactivities was evident in somata located along the inner one third of the retina. In addition, diffuse immunoreactivity was observed throughout the nascent IPL with both antibodies (Fig. 6A, B). At 20PN, ChAT- and SNAP-25-IRs within the INL were more distinct, and ChAT-immunoreactive processes began to segregate to sublamina 2 and 4 of the developing IPL (data not shown). At this age, the average number of somata immunoreactive for SNAP-25 and ChAT were similar, and the vast majority (90%) of SNAP-25-immunoreactive somata also exhibited ChAT-IR (Fig. 7), revealing their specific co-localization at this period of development.

The lamination pattern of the 25PN retina was more distinct than earlier ages. Choline acetyltransferase- and SNAP-25-immunoreactive cell bodies were restricted to the INL and GCL, and there was a further restriction of their processes to sublamina 2 and 4 of the IPL (Fig. 6C, D). At this age, there was a concomitant increase in the average numbers of ChAT and SNAP-25-immunoreactive cell bodies per section, with approximately 95% of SNAP-25-immunoreactive somata co-immunoreactive with the ChAT antibody (Fig. 7). At 30PN the retina displayed a marked increase in the average number of ChAT-immunoreactive cells per section (Fig. 7). Although the average number of SNAP-25-immunoreactive somata decreased, nevertheless, almost all (95.4%) were double-labeled with the ChAT antibody (Fig. 7). In the 35PN retina, the average number of ChAT-immunoreactive somata continued to increase (Fig. 7), and the immunoreactivity observed in sublamina 2 and 4 of the IPL was even more distinct (Fig. 6E). In contrast, the average number of strongly labeled SNAP-25-

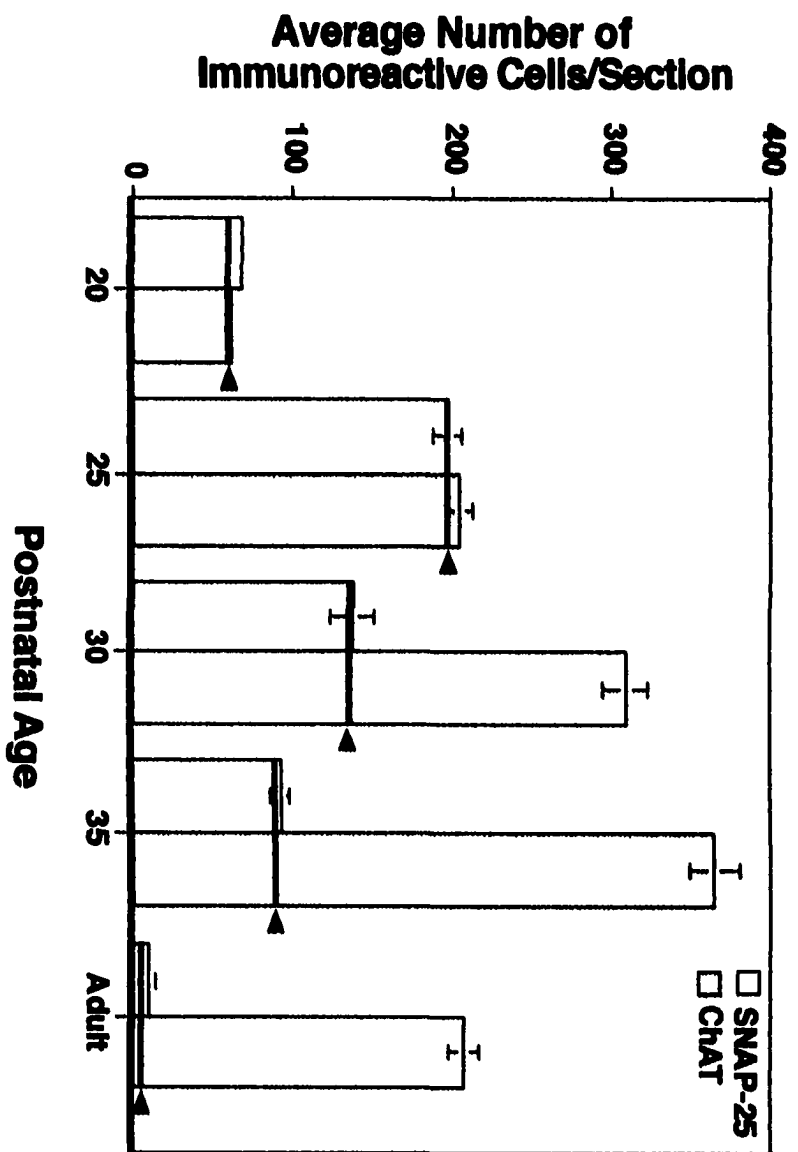
**Figure 5: Choline acetyltransferase-IR and SNAP-25-IR are co-localized in amacrine cells throughout the retina. Images from a 25PN retinal section starting from dorsal retina (A, B) to ventral retina (K, L). ChAT-IR (A, C, E, G, I, K) and SNAP-25-IR (B, D, F, H, J, L) are extensively co-localized throughout the dorso-ventral extent of the retina. Scale bar = 30  $\mu$ m.**



**Figure 6: Developmental distribution of ChAT-IR and SNAP-25-IR. Choline acetyltransferase-IR was first detected in the 15PN retina (A) shortly after high levels of SNAP-25-IR were first observed (B). In the 25PN retina, ChAT-IR (C) and SNAP-25-IR (D) were present in the same cells and processes in the IPL. At 35PN, the distinctive pattern of ChAT-IR (E) in cell bodies and processes was still evident, while high levels of SNAP-25-IR (F) in cell bodies were greatly diminished, though the bilaminar immunoreactivity in the IPL was still present. In the adult retina, ChAT-IR displayed its characteristic pattern (G), while SNAP-25-IR was now diffuse throughout the IPL and essentially absent from cell bodies (H). The arrowheads mark the region of retina that was analyzed by using density plot profiles illustrated in Figure 8. Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer. Scale bar = 20  $\mu$ m.**



**Figure 7: Transient localization of high levels of SNAP-25-IR within ChAT-immunoreactive amacrine cells during development. This graph represents the average number of ChAT-immunoreactive cell bodies and the average number of highly SNAP-25-immunoreactive cell bodies per section of retina from each age examined (see Materials and Methods). The horizontal axis represents the ages examined and the vertical axis represents the average number of highly SNAP-25-immunoreactive or ChAT-immunoreactive cell bodies per section. The arrowheads pointing to dark, horizontal lines, represent the average number of double-labeled cells per section. Error bars represent SEM. Sample sizes for each age are as follows: 20PN (n=12 sections from 4 eyes (12/4)); 25PN (n=17/6); 30PN (n=15/5); 35PN (n=9/3); Adult (n=6/2).**

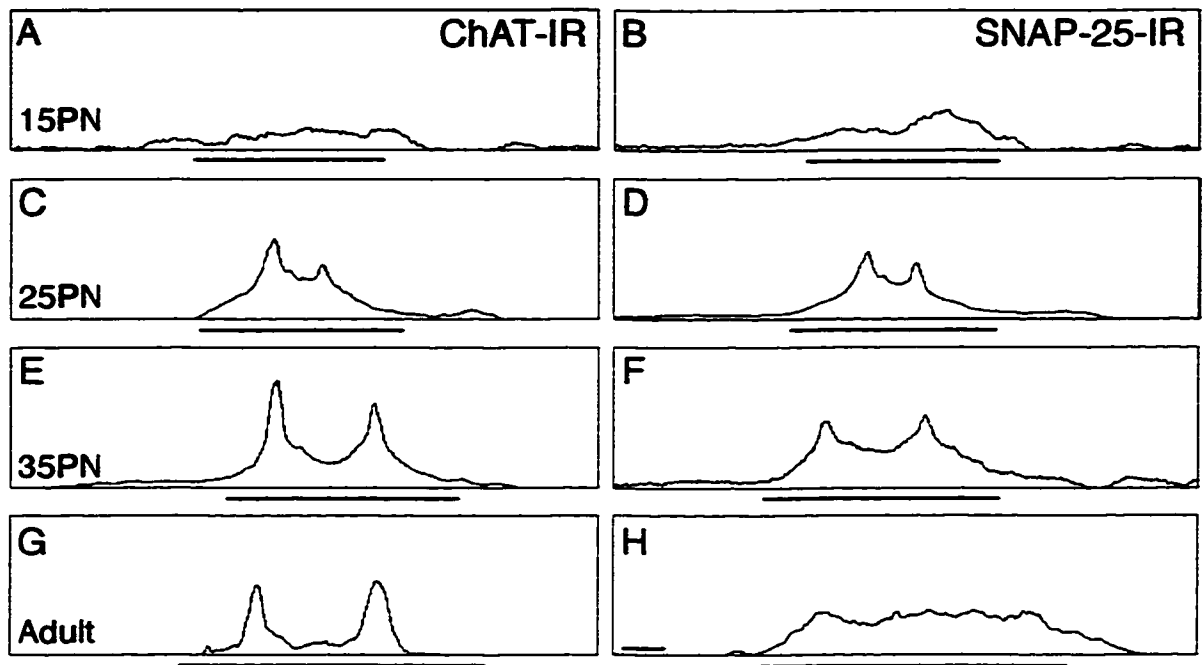




immunoreactive somata decreased (Fig. 7), although the intense immunoreactivity was still evident in sublamina 2 and 4 of the IPL (Fig. 6F). Despite the decrease in the average number of SNAP-25-immunoreactive somata, 91% were nevertheless ChAT-immunoreactive (Fig. 7).

In the adult retina, although the average number of ChAT-immunoreactive cells per section decreased (Fig. 7), ChAT-IR exhibited the same basic pattern as was observed in the 35PN retina (Fig. 6G). The decrease in the average number of ChAT-immunoreactive cells per retinal section may be due to cell death, or a change in the distribution of ChAT-immunoreactive cells as the retina continued to grow. In contrast, SNAP-25-IR in the adult retina differed significantly from the pattern of reactivity that was observed at earlier ages (Fig. 6H). Very few somata were immunoreactive for SNAP-25 (Fig. 7), and furthermore, SNAP-25-IR was diffuse throughout the IPL, lacking any obvious laminar pattern of labeling (Fig. 6H). These changing patterns of immunoreactivity observed in the IPL with the anti-ChAT and anti-SNAP-25 antibodies are illustrated using density plot profiles (Fig. 8). This densitometric analysis carried out on the images in Figure 6 (Fig. 6A-H; at the location marked with an arrowhead) clearly illustrates the dynamic changes in the characteristic patterns of ChAT and SNAP-25-IRs in sublamina 2 and 4 of the IPL (Fig. 8). Choline acetyltransferase-IR within the IPL was initially diffuse (Fig. 6A, 8A) and gradually became restricted to IPL sublamina 2 and 4 (Fig. 6C, E, G and 8C, E, G). In a similar fashion, SNAP-25-IR within the IPL was initially diffuse (Fig. 6B, 8B) and became restricted predominantly to sublamina 2 and 4 at 25 and 35PN (Fig. 6D, F and 8D, F). In contrast, in the adult, SNAP-25-IR was expressed in a diffuse pattern throughout the IPL (Fig. 6H and 8H). These results revealed a specific and transient co-localization of high levels of SNAP-25-IR in ChAT-immunoreactive amacrine cells. This discrete co-localization occurs during a dynamic period of retinal development during which cholinergic amacrine cells are likely to play a fundamental role in guiding the establishment of appropriate circuitry within the visual system.

**Figure 8: Densitometric analysis illustrating the changing levels of ChAT-IR (A, C, E, G) and SNAP-25-IR (B, D, F, H) within the IPL of the developing *Monodelphis* retina. These density plot profiles were taken from the images in Figure 6, at the location marked with arrowheads. Choline acetyltransferase-IR became more restricted to discrete sublamina within the IPL. In contrast, SNAP-25-IR within the IPL was initially diffuse (15PN), and became more restricted to sublamina 2 and 4 at 25 and 35PN. Finally, in the adult retina, SNAP-25 once again displayed a diffuse pattern of immunoreactivity. The solid line indicates the approximate region of the IPL at each age. Baseline levels were comparable to background fluorescence observed in unlabeled and secondary antibody controls. In each panel, plots from left to right represent outer to inner retina. Scale bar = 5 $\mu$ m.**



## Discussion

In the present study we have examined the development of cholinergic amacrine cells in the mammalian retina. Bromodeoxyuridine birthdating analysis revealed that ChAT-immunoreactive amacrine cells in the Brazilian opossum are born between 2 and 15PN, with the greatest proportion of cells being generated around 5PN. Using the anti-ChAT antibody and an antibody directed against SNAP-25 we have examined the spatial and temporal relationship of their patterns of expression. Our results revealed a specific and transient expression of relatively high levels of SNAP-25-IR in cholinergic amacrine cells during postnatal retinal development. This period in development began around 15PN, with the appearance of ChAT-IR within the same cells. In the 25PN retina, the greatest proportion of ChAT-immunoreactive amacrine cells displayed high levels of SNAP-25-IR. By 35PN, the approximate age of eye opening, most ChAT-immunoreactive amacrine cells no longer exhibited the intense SNAP-25-IR in their cell bodies, though prominent immunoreactivity was still observed in processes ramifying in sublamina 2 and 4 of the IPL. In the adult retina, high levels of SNAP-25-IR were no longer observed within ChAT-immunoreactive amacrine cells rather, there was diffuse, SNAP-25-IR throughout the IPL.

The intense immunoreactivity for SNAP-25 during retinal development was highly specific to cholinergic amacrine cells. Although a small percentage (< 5-10%) of the SNAP-25-immunoreactive somata lacked any obvious ChAT-IR, this may be due to the temporal difference in expression of SNAP-25, or our ability to detect higher levels of the protein, as low levels of SNAP-25-IR appear to precede detectable ChAT-IR during retinal development. Alternatively, the SNAP-25-immunoreactive neurons that lacked ChAT-IR may represent an additional, less populous, subclass of amacrine cells that also exhibits this differential expression of SNAP-25-IR during retinal development. Although the relatively high levels of SNAP-25-IR were observed within the inner retina, at younger ages SNAP-25-IR was also observed in the outer half of the retina, in the region of differentiating photoreceptors. The

significance of the lower levels of SNAP-25 expression in the outer retina has yet to be determined and may, perhaps, indicate a functional role for SNAP-25 during the differentiation of retinal cell types.

The functional requirement of SNAP-25 during transmitter release has been determined largely by studies using botulinum toxins (reviewed in Niemann et al., 1994). Other studies have defined additional roles for the protein during development (Osen-Sand et al., 1993; Bark et al., 1995; Osen-Sand et al., 1996), and possibly regeneration (Boschert et al., 1996). The spatial and temporal pattern of SNAP-25-IR in cholinergic amacrine cells, whose role during retinal development is presently under intense investigation (Feller et al., 1996; Sernagor and Grzywacz, 1996; Zhou and Fain, 1996), may afford a unique opportunity to further characterize the function of SNAP-25 *in vivo*.

Long before the onset of visual function, spontaneous activity is present in the developing vertebrate retina (Galli and Maffei, 1988; Meister et al., 1991; Wong et al., 1993; Feller et al., 1996). Blocking spontaneous activity prevents the normal segregation of ganglion cell axons into their appropriate eye-specific layers within the lateral geniculate nucleus (Shatz and Stryker, 1988). This correlated bursting activity is developmentally regulated, and subsides just prior to eye opening (Wong et al., 1993). Retinal activity prior to visual stimulation is important not only for refinement of the visual projection, but also for the establishment of appropriate circuitry within the retina itself. In the developing turtle retina, blocking cholinergic-dependent spontaneous retinal activity resulted in abnormally large retinal ganglion cell receptive fields (Sernagor and Grzywacz, 1996). The propagation and modulation of spontaneous retinal activity may be facilitated by several unique characteristics of cholinergic amacrine cells. Their relatively large arborizations might facilitate communication by a single amacrine cell with many ganglion cells and other amacrine cells, via both chemical and electrical synapses (Penn et al., 1994). In addition to acetylcholine, starburst amacrine cells release GABA, an inhibitory neurotransmitter (O'Malley et al., 1992). The co-release of an

excitatory and inhibitory neurotransmitter may contribute to the differential bursting activity which develops between On and Off ganglion cells as the retina continues to mature (Wong and Oakley, 1996). Furthermore, it is likely that the membrane excitability of the cholinergic amacrine cells changes during development. Zhou and Fain (1996) have recently shown in the rabbit retina that displaced starburst amacrine cells undergo a dramatic transition from spiking to nonspiking just after eye opening. Together, these results suggest that starburst amacrine cells may play an important role in the development of synaptic circuitry in the mammalian visual system.

The cholinergic-dependent spontaneous activity observed during the early development of the visual system may, in part, be regulated by differential expression of proteins which comprise the synaptic machinery necessary for regulated neurotransmitter release. One protein in particular, SNAP-25, appears to have two divergent roles in vesicle fusion events (Bark et al., 1995). SNAP-25 is crucial during regulated exocytosis of neurotransmitter (Bark and Wilson, 1994; Sudhof, 1995; Calakos and Scheller, 1996; Mehta et al., 1996) and may likely be involved in other vesicle fusion events during neurite extension (Osen-Sand et al., 1993; Catsicas et al., 1994; Osen-Sand et al., 1996). The transient and specific localization of high levels of SNAP-25 within ChAT-immunoreactive amacrine cells, suggests that it may be involved in regulating vesicular fusion events during the dynamic period of retinal development prior to eye opening. The relatively high levels of SNAP-25 may represent a selective enhancement of synaptic machinery in these neurons. Increased abundance of SNAP-25 may serve to facilitate the docking of a large number of synaptic vesicles with the presynaptic terminal membrane, thereby increasing the likelihood of transmitter release in response to an excitatory event. The role of SNAP-25 during docking of a synaptic vesicle involves its intimate association with other SNARE proteins (Bark and Wilson, 1994). In the *Monodelphis* retina immunoreactivity for the SNARE protein Syntaxin is present in the developing IPL as early as 10PN, and persists throughout development (Warne, West

Greenlee and Sakaguchi, unpublished observations). Furthermore, the synaptic terminal associated proteins Synaptotagmin, Rab3A and Synaptophysin are present throughout the developing IPL from 15PN into adulthood (West Greenlee et al., 1996). Thus, it appears that the synaptic machinery is present in the IPL such that high levels of SNAP-25 in ChAT-IR cells may be acting during release of neurotransmitter.

Alternatively, the high levels of SNAP-25 expression in ChAT-immunoreactive amacrine cells may also be involved in mediating distinct vesicle-membrane fusion events during neurite extension. Synaptosomal Associated Protein-25 protein has been shown to be necessary for neurite outgrowth during development (Osen-Sand et al., 1993; Osen-Sand et al., 1996). These studies implicate a role of SNAP-25 in the docking and fusion of plasmalemma precursor vesicles at the growing neurite tip (Osen-Sand et al., 1996). The distinction between two divergent vesicle fusion events leading to neurotransmission and neurite extension may be mediated by different isoforms of SNAP-25 (i.e. SNAP-25a and SNAP-25b; Bark et al., 1995). These isoforms appear to be developmentally and spatially regulated. SNAP-25a is present predominantly during development and regeneration (Bark et al., 1995; Boschert et al., 1996), while SNAP-25b expression appears to coincide with synaptic maturation (Bark et al., 1995). Due to the differential localization and presumed functions of the two SNAP-25 isoforms, studies to identify which isoform (s) comprise the high levels in ChAT-immunoreactive amacrine cells may ultimately lead to a better understanding of their functional significance. Furthermore, high levels of SNAP-25 may be involved during the establishment of synaptic circuitry in ChAT-immunoreactive amacrine cells. Previous studies by Osen-Sand et al., (1996) demonstrated that cleavage of SNAP-25 with botulinum toxin inhibited synapse formation in developing central neurons. Thus the period of high SNAP-25 expression in ChAT-immunoreactive neurons may represent a period of extensive synaptogenesis and/or synaptic plasticity for these cells.

In addition to the *Monodelphis* retina, we have recently found that relatively high levels of SNAP-25-IR are specifically localized to ChAT-immunoreactive cells in the postnatal rat retina prior to eye opening (6PN; West Greenlee and Sakaguchi, unpublished observations). These results provide evidence that high levels of SNAP-25 in ChAT-immunoreactive amacrine cells are likely to be a ubiquitous phenomenon required for cholinergic-dependent processes during mammalian retinal development.

It is likely that cholinergic amacrine cells mediate events crucial for the establishment of proper retinal circuitry, and therefore, would influence further development of the mammalian visual system. The specificity and time course of the expression of high levels of SNAP-25-IR in cholinergic amacrine cells provides evidence that this presynaptic terminal-associated protein may play an integral role in mediating the transient function provided by these amacrine cells during this critical period of development. Further studies should reveal whether additional aspects of membrane trafficking and vesicle fusion, facilitated by SNAP-25, underlie the specific needs for the developmental plasticity of the nervous system.

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# **DIFFERENTIAL LOCALIZATION OF SNARE COMPLEX PROTEINS SNAP-25, SYNTAXIN, AND SYNAPTOBREVIN DURING DEVELOPMENT OF THE MAMMALIAN RETINA**

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M.H. West Greenlee<sup>1,2</sup>, C.B. Roosevelt,  
and D.S. Sakaguchi<sup>1,3</sup>.

## **Abstract**

In this analysis, we have examined the differential localization of three SNARE complex proteins during differentiation of the mammalian retina. Using antibodies directed against SNAP-25, Syntaxin and Synaptobrevin/VAMP, we characterized their changing patterns of immunoreactivity during retinal differentiation in both Brazilian opossum and rat. We observed temporal differences in the onset of immunoreactivity between these three proteins, and differences in their localization in cellular and synaptic layers in the retina. In addition, though immunoreactivity for SNARE complex proteins was co-localized throughout much of the retina, their staining patterns exhibited characteristic variances in staining intensity within these regions both during development and at maturity. The differential distribution of SNAP-25, Syntaxin, and VAMP may indicate additional roles for these proteins during vesicle trafficking events, which are independent of SNARE complex formation.

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<sup>1</sup> Graduate student and Associate Professor, respectively. Department of Zoology and Genetics, Neuroscience Program, Iowa State University

<sup>2</sup> Primary researcher and author

<sup>3</sup> Author for correspondence

## Introduction

The release of neurotransmitter at the synapse is a highly regulated process involving fusion of transmitter-filled synaptic vesicles with the presynaptic terminal membrane (Calakos and Scheller, 1996). The SNARE hypothesis is a model which proposes that docking of vesicles at the target, presynaptic membrane is achieved via the interaction of a membrane protein on the vesicle (v-SNARE) with two proteins at the target membrane (t-SNAREs) thereby forming the SNARE complex (Sollner *et al.*, 1993). This SNARE complex then serves as a binding site for other cytosolic and membrane bound proteins which facilitate ATP-hydrolysis, regulated vesicle fusion and subsequent release of transmitter (Bark and Wilson, 1994b; Calakos and Scheller, 1996; Südhof, 1995). In this way, Synaptobrevin, or Vesicle associated Membrane Protein (VAMP), the v-SNARE, and the t-SNAREs Syntaxin and Synaptosomal Associated Protein of 25 kDa (SNAP-25) form a backbone whereby interacting and associated proteins may affect their association with each other and other proteins to regulate this critical cellular trafficking event (Calakos and Scheller, 1996; Fujita *et al.*, 1998).

In addition, proteins of the SNARE complex may function during development and regeneration. Disruption of SNAP-25 expression using anti-sense oligonucleotides, or cleavage of the protein with botulinum toxin A or E, disrupts neurite outgrowth and synaptogenesis during development (Osen-Sand *et al.*, 1993; Osen-Sand *et al.*, 1996). These results suggest that fusion of plasmalemma precursor vesicles, membranous vesicles unique to growth cones (Pfennitter *et al.*, 1992), with the tip of the growing neurite may require SNAP-25 (Catsicas *et al.*, 1994; Osen-Sand *et al.*, 1993; Osen-Sand *et al.*, 1996). Syntaxin may also be critical for neurite extension during development. Igarashi *et al.* (1996), demonstrated that cleavage of Syntaxin with botulinum toxin C1, disrupted neurite outgrowth. Using peptides containing a critical portion of the site where Syntaxin interacts with VAMP, they were also able to induce growth cone collapse and inhibit neurite outgrowth. These results suggest that Syntaxin's role during neurite outgrowth involves fusion of plasmalemma precursor vesicles,

through the formation of a SNARE-like complex (Igarashi *et al.*, 1996). However, experiments which used botulinum B or tetanus toxin to cleave VAMP during development did not affect neurite outgrowth and synaptogenesis (Osen-Sand *et al.*, 1996).

The mammalian retina has served as an excellent model in which to investigate the developmental expression of presynaptic terminal-associated proteins (Catsicas *et al.*, 1992; Karne *et al.*, 1997; Osen-Sand *et al.*, 1996; West Greenlee *et al.*, 1998; West Greenlee *et al.*, 1996). The retina is a highly organized CNS structure and undergoes a characteristic pattern of differentiation (Altshuler *et al.*, 1991). It has a laminar organization, consisting of three cellular layers, separated by two synaptic layers (Rodiek, 1973). Within these synaptic layers reside three morphologically different types of synapses. In the outer plexiform layer (OPL), there are ribbon synapses formed by photoreceptors (Sjöstrand, 1953) and non-vesicular synapses from horizontal cells (Schwartz, 1987). Ribbon synapses from bipolar cells (Kidd, 1962) and conventional synapses from amacrine cells (Mandell *et al.*, 1990) compose the synaptic profiles found within the inner plexiform layer (IPL). In addition to the synaptic diversity of the retina, there is considerable biochemical diversity. Amacrine cells are the most diverse type of retinal neuron, with over 20 subtypes identified so far, based on their primary neurotransmitter (Masland, 1996; Pourcho, 1996). Each amacrine cell subtype has a distinctive arborization pattern of their processes in the IPL. The sum of these arborizations has been used to define the five sublamina of this synaptic layer (Karten and Brecha, 1983; Marc, 1986).

During development, cells of the retina participate in activity dependent events which are necessary for establishing proper circuitry in the retina, and the rest of the visual system. Glutamate-dependent activity is required for proper segregation of retinal ganglion cell (RGC) dendrites into ON and OFF IPL sublamina, the inner 3/5 and the outer 2/5, respectively (Bodnarenko and Chalupa, 1993; Bodnarenko *et al.*, 1995). In addition, within the developing retina spontaneous, patterned activity, mediated by cholinergic synaptic transmission (Feller *et*



*et al.*, 1996), is essential for proper establishment of RGC receptive fields (Semagor and Grzywacz, 1996) and segregation of RGC terminals into eye specific lamina in the lateral geniculate nucleus (LGN) (Penn *et al.*, 1998). Furthermore, the subsequent modulation of this patterned activity correlates with segregation of RGC terminals into ON and OFF sublamina in the LGN (Fisher *et al.*, 1998; Wong and Oakley, 1996). Analysis of the changing distribution of SNARE complex proteins in relation to the dynamics of spontaneous activity in the developing retina, and the differentiation of laminar organization may be directive for further, more functional studies of SNARE complex proteins during development.

In the current study, we have examined the developmental distribution of SNARE complex proteins during development of two mammalian retinæ; the rat and the Brazilian opossum, *Monodelphis domestica*. Extensive characterization of the developing rat retina has been done at the physiological (Galli and Maffei, 1988), biochemical (Cellerino *et al.*, 1998; Mitrofanis *et al.*, 1988; Puro *et al.*, 1982), anatomical (Alexiades and Cepko, 1997; Kahn, 1974; Reese *et al.*, 1992) and ultrastructural (Weidman and Kuwabara, 1968) levels. Thus, there is a significant basis to compare presynaptic terminal protein localization to other events in the developing rat retina.

In order to more accurately resolve the developmental changes in localization of SNARE complex proteins, we took advantage of the protracted period of postnatal retinal development of the Brazilian opossum, *Monodelphis domestica*. (Kuehl-Kovarik *et al.*, 1995; West Greenlee *et al.*, 1998; West Greenlee *et al.*, 1996). At birth, the *Monodelphis* retina is a relatively undifferentiated neuroepithelium with the earliest differentiating ganglion cells in dorso-central retina (West Greenlee *et al.*, 1996). Eye opening and visual function, based on the presence of a menace response (West Greenlee and Sakaguchi, unpublished observation) occur around 35 days postnatal (35PN). Their immaturity at birth, and their protracted period of postnatal development makes marsupials, like *Monodelphis*, excellent models for the study of visual

system development (Cavalcante, 1985; Dunlop *et al.*, 1996; Dunlop *et al.*, 1997; Harman *et al.*, 1992; MacLaren and Taylor, 1995; Mark *et al.*, 1993).

In this analysis, we have observed differences in the time course and relative levels of SNARE protein distribution in the developing mammalian retina. These proteins which function together during transmitter release may also have functional importance independent of one another during retinal differentiation. Their differential localization and regulation during development may be important for the appropriate formation of retinal circuitry.

## **Methods and Materials**

### **Animals**

Brazilian opossums were obtained from a colony maintained at Iowa State University. The opossums were maintained in a constant temperature environment (26°C) on a 14:10 hour light:dark cycle with food (Reproduction Fox Chow; Milk Specialties Products, Madison, WI) and water *ad libitum*. The Brazilian opossum has a gestation period of approximately 13.5 days. Date of birth was designated as postnatal day 1 (1PN). Pups were weaned from the mother at 60PN.

Pregnant rats were obtained from Harlan Sprague Dawley (Indianapolis, IN). Females and subsequent pups were maintained by Laboratory Animal Resources at Iowa State University until they were sacrificed. Laboratory procedures were carried out in accordance with guidelines, and had the approval of the Iowa State University committee on animal care.

### **Tissue Preparation**

We have examined retinal development in opossum pups at postnatal days 1, 5, 10, 25, and 35 and rat pups at postnatal ages 1, 5, 7, 10 and 15 as well as in adult animals. Opossum pups between 1 and 10PN were anesthetized by hypothermia. The animals were then decapitated and their heads fixed by immersion in 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) in 0.1M sodium phosphate buffer (pH 7.5) for 48 hours. All other animals

were deeply anesthetized with ether and perfused transcardially with 4% paraformaldehyde. The heads (25 and 35PN opossums, 1-10PN rats) or eyes (removed from the head in all older animals) were then post-fixed for 48 hours. Tissue was cryoprotected in a 30% sucrose solution in 0.1 M PO<sub>4</sub> buffer, and sectioned coronally at 20 µm on a cryostat (American Optical). Sections were thaw mounted onto Superfrost (Fisher) microscope slides and stored at 4° C or -20° C until processed. At least three retinæ, from two animals of different litters were used in the analysis for each developmental time point examined.

### **Immunohistochemistry**

The protocol utilized for immunohistochemistry was a modification of that previously described by Elmquist and colleagues (1992). Slide mounted tissue sections were rinsed in potassium phosphate buffered solution (KPBS; 0.15 M NaCl, 0.034 M K<sub>2</sub>HPO<sub>4</sub>, 0.017 M KH<sub>2</sub>PO<sub>4</sub>, pH 7.4), and endogenous peroxidase activity eliminated by a 30 minute incubation in 0.3% hydrogen peroxide solution in KPBS. The sections were then incubated for two hours in blocking solution (KPBS with 1% bovine serum albumin (BSA; Sigma, St. Louis, MO), 0.4% Triton X-100 (Fisher), and 1.5% normal blocking serum (NBS, horse or goat; Vector Laboratories Inc., Burlingame, CA), and incubated overnight at room temperature (23°C) in the respective primary antibodies (see below). On the following day, tissue sections were rinsed in KPBS with 0.2% Triton X-100 and incubated in an appropriate biotinylated secondary antibody for two hours at room temperature, rinsed, and incubated in Horseradish Peroxidase-Avidin-Biotin Complex for 1 hour at room temperature (Vector Elite ABC Kit; 1:200 or 1:600; Vector). In order to visualize the antibody staining pattern, the tissue was reacted with a substrate of 0.4% 3',3' diaminobenzidine tetrahydrochloride (DAB; Sigma), 2.5% nickel sulfate (Sigma) and 0.05% hydrogen peroxide (Fisher), in 0.1 M sodium acetate (Fisher). The reaction was terminated in successive rinses of 0.9% NaCl solution. Sections were then dehydrated through a graded ethanol series, cleared with xylene, and coverslipped with Accumount (Baxter Scientific, McGaw Park, IL).

### **Triple label analysis**

Slide mounted tissue sections were rinsed in 0.5M KPBS, incubated for 2 hours in blocking solution, and incubated overnight at 24°C in anti-SNAP-25 (see below). On the following day, tissue sections were rinsed in KPBS with 0.2% Triton X-100 and incubated in biotinylated secondary antibody (see below). To visualize the antibody staining pattern, sections were then incubated in Cy3 conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA; 1:5000) for 30 minutes. After thorough rinsing, tissue sections were incubated in blocking solution (PBS: 14 mM NaCl, 2.7 mM KCl, 5.37 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.76 mM KH<sub>2</sub>PO<sub>4</sub>, pH7.4; with 5% goat serum, 0.4% BSA and 0.2% Triton X-100) for 30 minutes and incubated in diluted anti-VAMP (see below) at 4°C overnight. After washes in PBS with 0.25% Triton X-100, VAMP-IR was visualized with Alexa 488 (Molecular Probes, Eugene, OR) conjugated goat anti-mouse IgM. After subsequent rinsing in PBS, sections are incubated in blocking solution for 30 minutes and anti-Syntaxin (see below) at 4°C overnight. Sections were then washed in PBS with 0.25% Triton X-100, Syntaxin-IR was visualized with Cy5 conjugated goat anti-mouse IgG (Jackson ImmunoResearch). After subsequent washes in PBS, sections were coverslipped with Vectashield fluorescence mounting medium (Vector).

Negative controls were run in parallel during the immunohistological processing by the omission of the primary or secondary antibodies. No antibody staining was observed in the controls.

### **Antibodies**

Anti-Synaptosomal-Associated Protein-25 (SNAP-25) antisera (M.C. Wilson, or StressGen Biotechnologies, Victoria, BC Canada) was raised against a synthetic peptide corresponding to the carboxyl 12 residues of the mouse protein (Oyler et al., 1989) and was diluted at 1:2000. The anti-Syntaxin antibody, HPC-1 (Barnstable et al., 1985) (Sigma) was diluted 1:5000. Anti-VAMP (or Synaptobrevin) (StressGen) was diluted 1:200. All primary

antibodies were diluted in KPBS with 1% BSA, 0.4% Triton X-100, and 1% NBS.

Biotinylated donkey-anti-rabbit, donkey anti-mouse IgG, and goat-anti-mouse IgM secondary antibodies (Jackson ImmunoResearch) were diluted at 1:600. All secondary antibodies were diluted in KPBS with 1% BSA, 0.02% Triton X-100, and 1% NBS. The specificity of the SNAP-25 antisera has been previously examined in immunoblot analysis, with the antibody detecting a specific band with an apparent molecular weight of approximately 25 kDa (Swanson *et al.*, 1996).

### **Analysis of Tissue Sections**

Tissue sections were examined with a Nikon Microphot FXA photomicroscope (Nikon Corp., Tokyo, Japan). Images were captured using a Kodak Megaplug Camera (Model 1.4; Kodak Corp., San Diego, CA) connected to a Perceptics MegaGrabber framegrabber in a Macintosh 8100/80AV computer (Apple Computer, Cupertino, CA) using NIH Image 1.55VDM software (Wayne Rasband, National Institutes of Health, Bethesda, MD obtained at FTP site [zippy.nimh.gov](http://zippy.nimh.gov)). Analysis of triple-labeled sections was done with a Leica TCS-NT confocal scanning laser microscope (Leica Microsystems, Inc., Exton, PA).

Density plot profiles were prepared by using NIH Image software. For this analysis, a profile line plot was obtained from digitized images of immunostained tissue sections of adult retina in Figures 1-6F. Each profile line corresponded to a strip 20 $\mu$ m wide of immunostained IPL. This method of analysis provides information about relative intensities of immunostaining. Although this approach does not provide evidence for differences in absolute levels of expression of proteins of interest, this strategy allows a comparison of the relative levels of expression within different sublamina of the IPL (Catsicas *et al.*, 1992; West Greenlee *et al.*, 1998). Figures were prepared using Adobe Photoshop Version 4.0 and Macromedia Freehand Version 5.5 for the Macintosh. Outputs were generated on a Tectronix phaser continuous tone color printer (Tectronix, Beaverton, OR).

## Results

Using standard immunohistological techniques, we examined the differential localization of SNARE complex proteins in the developing mammalian retina. Differences in the temporal onset of detectable immunoreactivity, and relative intensities within differentiating cellular and synaptic layers suggest differential regulation of these proteins during development. This differential regulation may be critical for retinal differentiation and establishment of appropriate retinal circuitry.

The most significant differences in SNARE complex protein distribution between *Monodelphis* and rat were observed in the differentiating IPL. Thus, patterns of immunoreactivity for SNARE complex proteins in the IPL will be presented separately for each species, while presentation of their patterns of immunoreactivity in the rest of the retina will be combined.

### **SNARE proteins are differentially localized in the differentiating *Monodelphis* IPL**

At birth (1PN) the *Monodelphis* retina is relatively undifferentiated. SNAP-25-like immunoreactivity (-IR) was present in newly differentiating cell bodies and processes in dorso-central retina (Fig. 1A). Similarly, Syntaxin-IR was observed in the axons of newly differentiated ganglion cells exiting the eye at the optic nerve head (Fig. 2A). Faint, diffuse VAMP-IR was detected in cell bodies in central retina (Fig. 3A). At 5PN, there was still no obvious laminar organization, though immunoreactivity for all three SNARE proteins was detectable in the inner region of the retina (Figs 1, 2, 3 B). By 10PN, evidence of IPL formation was evident. SNAP-25 and Syntaxin-IRs were detected in this nascent IPL, while VAMP-IR was faintly detected in some, but not all preparations. By 25PN, still 10 days prior to eye opening, SNAP-25-IR displayed a very distinctive pattern. SNAP-25-IR was present throughout the IPL, with considerably more intense-IR in sublamina 2 and 4 (Fig. 1D). Previous studies have demonstrated that this intense SNAP-25 labeling is present in processes

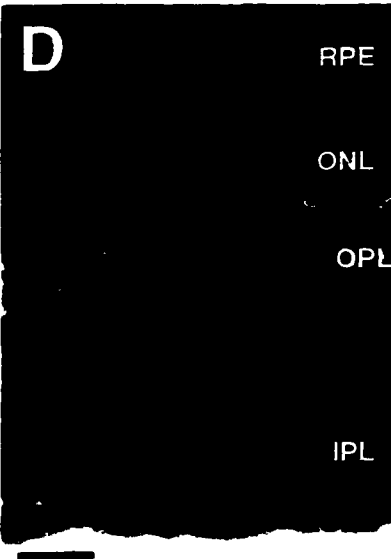
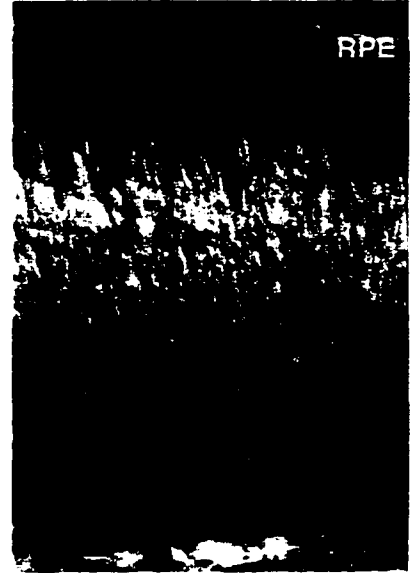
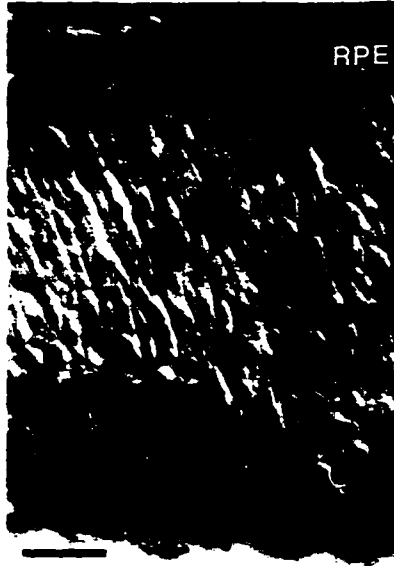
of starburst amacrine cells (West Greenlee et al., 1998). In contrast, immunoreactivity for Syntaxin and VAMP was relatively uniform throughout the IPL (Figs. 2 and 3D). At 35PN, around the time of eye opening, the intense SNAP-25-IR in sublamina 2 and 4 had decreased, but was still present (Fig. 1E), Syntaxin-IR was still relatively uniform, though the outer margin of the layer was demarcated by slightly more intense immunoreactivity (Fig. 2E). VAMP-IR was also relatively uniform, though the inner margin of the layer was demarcated by intense, punctate immunoreactivity (Fig. 3E). The mature *Monodelphis* IPL also displayed differential SNARE protein immunoreactivity. Figure 7 illustrates the differential intensity of the three proteins in the IPL using density plot profile analysis (see Materials and Methods). SNAP-25-IR in the IPL at this age was relatively uniform (Fig. 1F). In contrast, Syntaxin-IR was non-uniform, with many small unlabeled regions. In addition, the inner and outer limits of this layer are demarcated by intense immunoreactivity (Fig. 2F). VAMP-IR was present throughout the extent of the adult IPL, and what appeared to be sublamina 5 was demarcated by more intense, punctate, VAMP-IR (Fig. 3F).

### **SNARE proteins are differentially localized in the developing rat IPL**

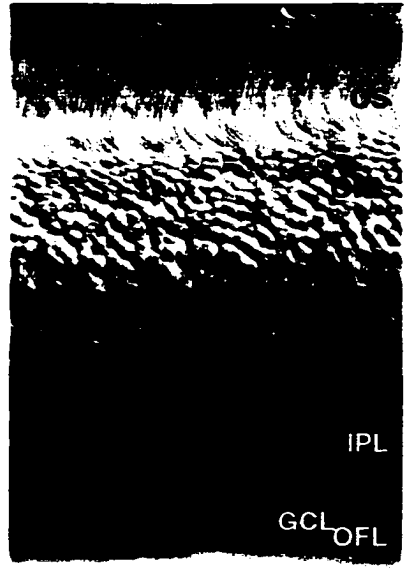
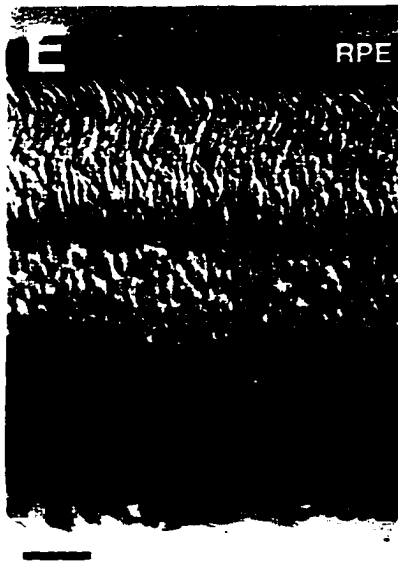
At birth, the rat retina was significantly more mature than the opossum retina. The developing IPL was immunoreactive for all three SNARE proteins (Fig. 4,5,6A). At 5PN, SNAP-25-IR in the rat retina was similar to that observed in a 20PN opossum retina (data not shown) with considerably more intense labeling of sublamina 2 and 4 (Fig. 4B). In contrast, Syntaxin- (Fig. 5B) and VAMP-IRs (Fig. 6B) were relatively uniform throughout the IPL at this stage of development. Two days later, at 7PN, SNAP-25-IR in the IPL was still most intense in sublamina 2 and 4, but the inner limit of this layer was also beginning to display a more intense immunoreactivity (Fig. 4C). Syntaxin-IR in the IPL was still relatively uniform (Fig. 5C), while VAMP-IR displayed a tri-laminar pattern and was most intense in sublamina 1, 3 and 5 (Fig. 6C). As IPL differentiation progressed, the intensity of SNAP-25-IR

**Figure 1: SNAP-25-immunoreactivity in the postnatal opossum retina. At 1PN, SNAP-25-IR is present in dorso-central retina (A). In the 5PN opossum retina, SNAP-25-IR was detected in the inner retina, and cells in the outer retina adjacent to the RPE (B). SNAP-25-IR in the 10PN retina was detected in the inner retina including the nascent IPL, and in more cells in the outer retina (C). By 25PN, SNAP-25-IR was detected throughout the retina. Relatively more intense immunoreactivity was detected in a subset of cells in the INL and GCL, with immunoreactive processes in sublamina 2 and 4 respectively. Cells in the outer third of the retina were also more intensely immunoreactive with an antibody against SNAP-25. (D). Ten days later, SNAP-25-IR in the 35PN retina was similar to that observed in the 25PN retina but the intensely immunoreactive cell bodies in the INL and GCL were much less so (E). Immunoreactivity for SNAP-25 in the mature, adult retina was most intense in fiber layers and in the outer two-thirds of the ONL and in the inner region of the outer segments (F). Scale bars; A = 50 $\mu$ m; B, C, D, E = 30 $\mu$ m; F 20 $\mu$ m. Abbreviations: ONH, optic nerve head; OFL, optic fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer, OS, outer segments; RPE, retinal pigment epithelium.**

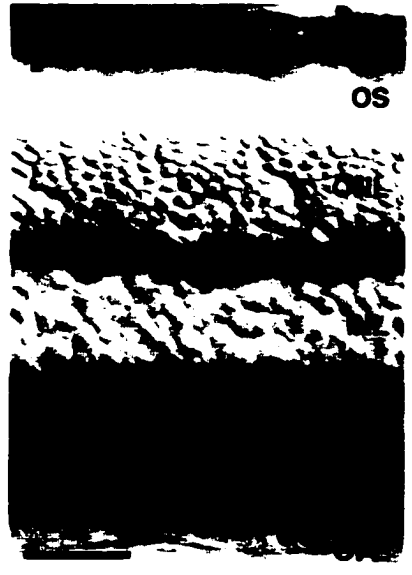
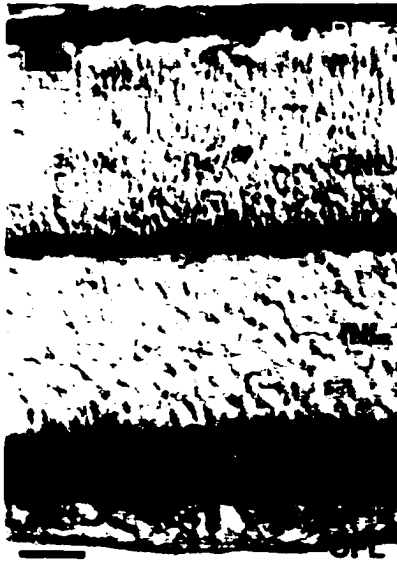
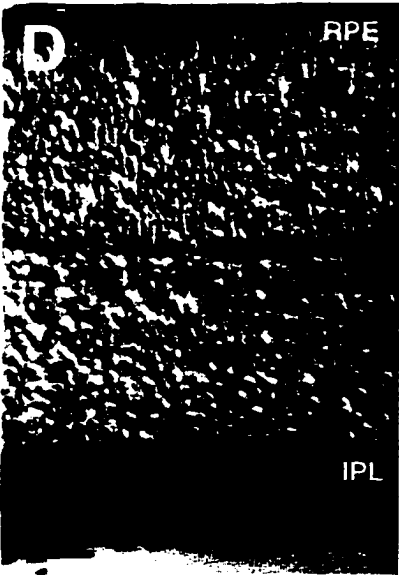
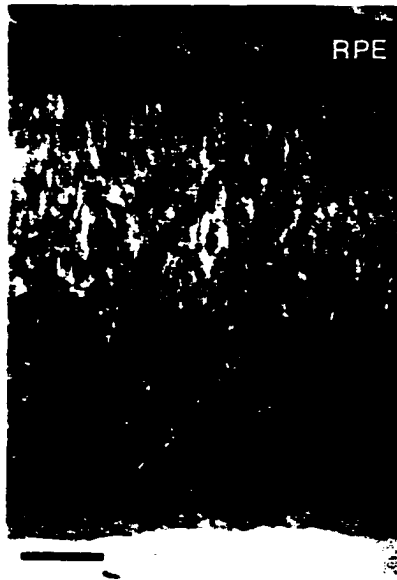




**Figure 2: Syntaxin-immunoreactivity in the postnatal opossum retina. At 1PN, Syntaxin-IR is present in dorso-central retina (A). In the 5PN opossum retina, Syntaxin was detected in the inner retina (B). Syntaxin-IR in the 10PN retina was detected in the inner retina in the OFL and nascent IPL (C). By 25PN, Syntaxin-IR was present in the OFL, IPL, and faintly in the OPL. In addition, immunoreactivity was detected in cells in the inner one-half of the INL (D). Ten days later, Syntaxin-IR in the 35PN retina was similar to that observed in the 25PN retina (E). Immunoreactivity for Syntaxin in the adult retina was most intense in fiber layers, and in the INL. Immunoreactivity in the IPL was most intense at the inner and outer border of this layer (F). Scale bars; A = 50µm; B, C, D, E = 30µm; F 20µm. Abbreviations: ONH, optic nerve head; OFL, optic fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer, OS, outer segments; RPE, retinal pigment epithelium.**



**Figure 3: VAMP- like-immunoreactivity in the postnatal opossum retina. At 1PN, VAMP-IR is present in dorso-central retina (A). In the 5PN opossum retina, VAMP-IR was detected in the inner retina, and cells in the outer retina adjacent to the RPE (B). SNAP-25-IR in the 10PN retina was diffuse in the inner retina (C). By 25PN, VAMP-IR was limited to the OFL and IPL. VAMP-IR was not yet detectable in the OPL (D). Ten days later, VAMP-IR in the 35PN retina was similar to that observed in the 25PN retina but immunoreactivity was now detectable in the OPL (E). Immunoreactivity for VAMP in the adult retina was present in the IPL and OPL. VAMP-IR was most intense in the inner one-fourth of the IPL (F). Scale bars; A = 50µm; B, C, D, E = 30µm; F 20µm. Abbreviations: ONH, optic nerve head; OFL, optic fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer, OS, outer segments; RPE, retinal pigment epithelium.**

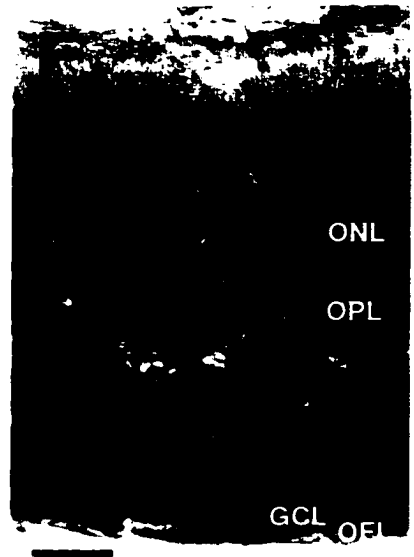
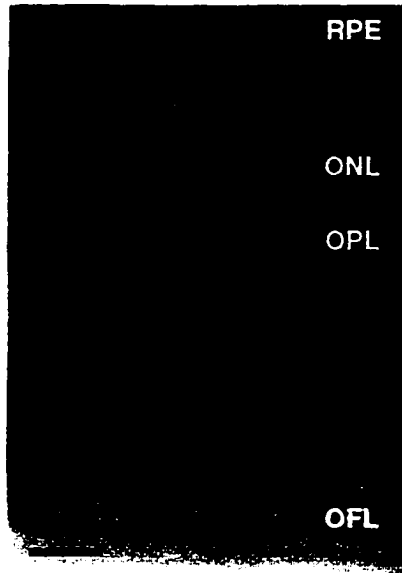
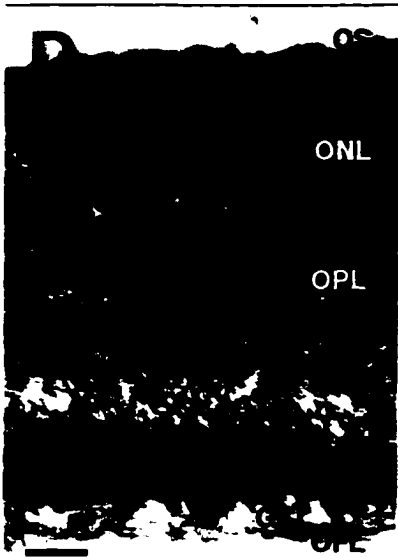
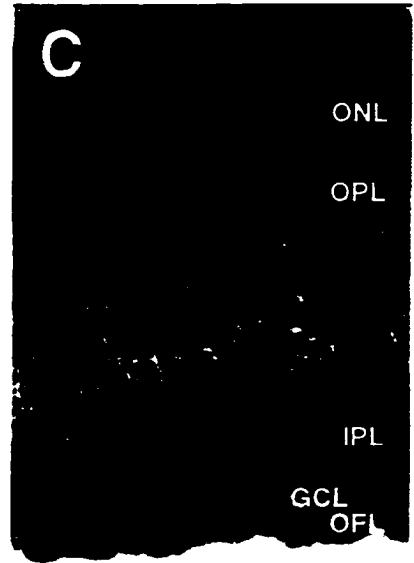
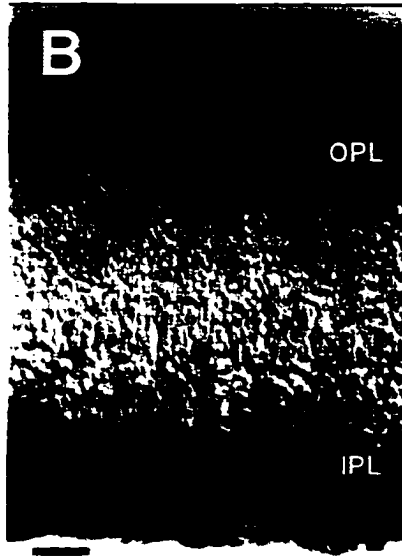
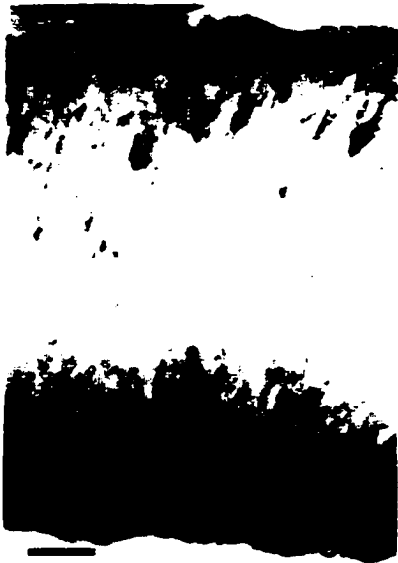


increased in sublamina 1 and 5. At 10PN, the intense SNAP-25-IR divided the IPL into 3 regions, an outer region of intense immunoreactivity (sublamina 1 and 2), a middle region of relatively less immunoreactivity (sublamina 3) and an inner region of more intense immunoreactivity (sublamina 4 and 5). Syntaxin-IR at this age of development had begun to resemble the adult pattern. Syntaxin-IR was present throughout the IPL, with the inner and outer margins demarcated with more intense labeling (Fig. 5D). VAMP-IR at this age also resembled the adult pattern. VAMP-IR was detected throughout the IPL, and the inner one-fourth of the IPL (roughly corresponding to sublamina 5) was more intensely immunoreactive (Fig. 6D). There was little change in the localization of SNARE proteins in the IPL between 10 and 15PN (Figs. 4, 5, and 6). In the adult rat retina, the three SNARE proteins also exhibited differential intensities throughout the IPL (Fig. 7). SNAP-25-IR was present throughout the IPL, with the most intense labeling in sublamina 5 (Fig. 4F), Syntaxin-IR (similar to the opossum) was most intense at the inner and outer borders of this layer (Fig. 5F), and VAMP-IR was most intense in the inner or 5th sublamina (Fig. 6F).

### **Differential localization of SNARE proteins in the developing OPL**

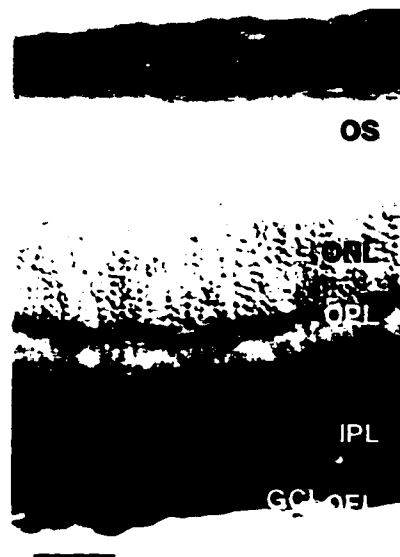
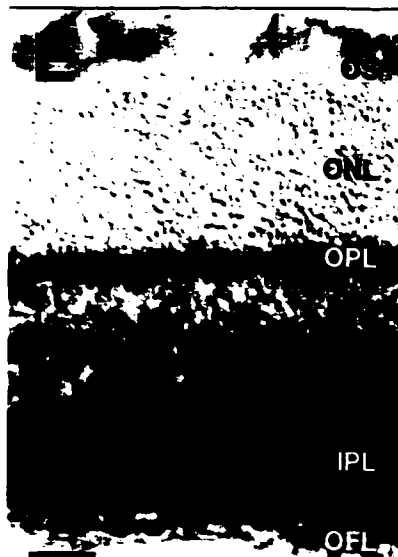
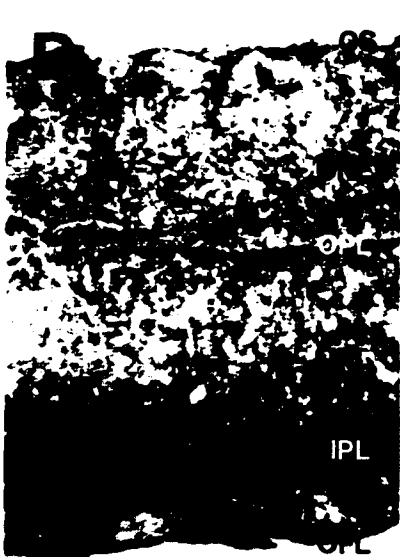
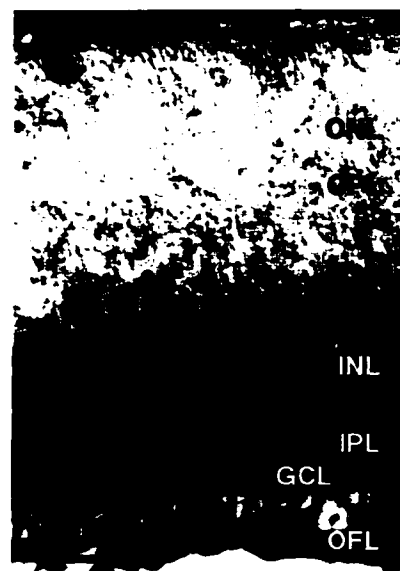
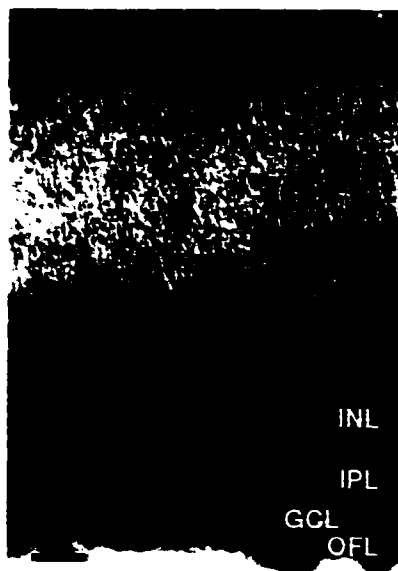
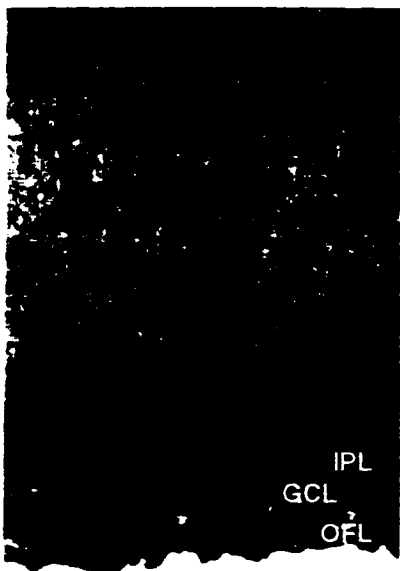
In the *Monodelphis* retina, the OPL is morphologically identifiable around 25PN (Figs 1, 2, 3D). SNAP-25-IR was detected throughout the OPL at this age (Fig. 1D) and in the mature retina (Fig. 1 E, F). Syntaxin-IR was also detected in the OPL at this age, but the label was faint, and restricted to the inner half of this layer (Fig. 2D, E and F). VAMP-IR in the OPL was not detected until 35PN (Fig. 3E). At this age and in the mature retina (Fig. 3F), VAMP-IR was restricted to the outer half of the OPL. In the developing rat retina, the OPL was morphologically detectable by 7PN, and patterns of immunoreactivity for all three SNARE complex proteins were similar to what was observed in *Monodelphis* (Fig. 4,5 and 6C). Confocal-scanning laser microscopy was performed on triple-labeled preparations of 35PN *Monodelphis* (data not shown) and 15PN rat retina to examine the differential localization of

**Figure 4: SNAP-25-immunoreactivity in the postnatal rat retina. At 1PN, SNAP-25-IR was present in the inner retina in the OFL, GCL, differentiating IPL and INL. In addition, cell bodies in the outer retina (A). In the 5PN rat retina, SNAP-25-IR was detected in the inner retina in the OFL, GCL, IPL, and INL as well as the differentiation OPL and all cells to the outside of this layer. SNAP-25-IR in the inner retina, was relatively more intense in a subset of cells in the INL and GCL with processes in sublamina 2 and 4 of the IPL (B). SNAP-25-IR in the 7PN retina was similar to that observed in the 5PN retina, though the intensity of SNAP-25-IR in INL and GCL cells was much more uniform (C). By 10PN, SNAP-25-IR was detected almost throughout the retina with the exception of the middle one-third of the INL. Within the IPL, relatively intense SNAP-25-IR was still observed in sublamina 2, the pattern in sublamina 4 was more difficult to distinguish due to the increase of immunoreactivity vitreal to this sublamina (D). In the 15PN rat retina, SNAP-25-IR was detected throughout the retina. Immunoreactivity in the IPL was more intense outside of sublamina 2 and inside of sublamina 4 (E). Immunoreactivity for SNAP-25 in the mature, retina was most intense in fiber layers and in the outer two-thirds of the ONL and in the inner region of the outer segments. The inner one-fourth of the IPL was more intense than the rest of this layer (F). Scale bars; A ,E = 40µm; B, D = 30µm; C, F = 50µm. Abbreviations: ONH, optic nerve head; OFL, optic fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer, OS, outer segments; RPE, retinal pigment epithelium.**



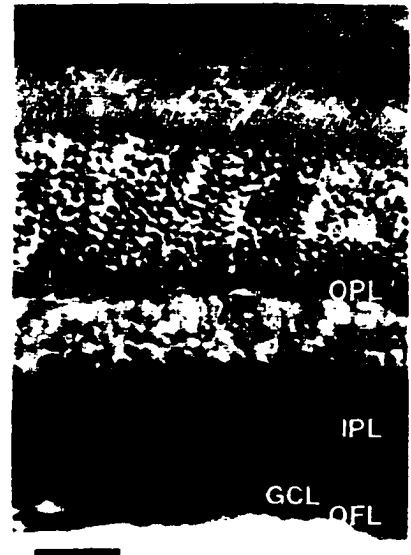
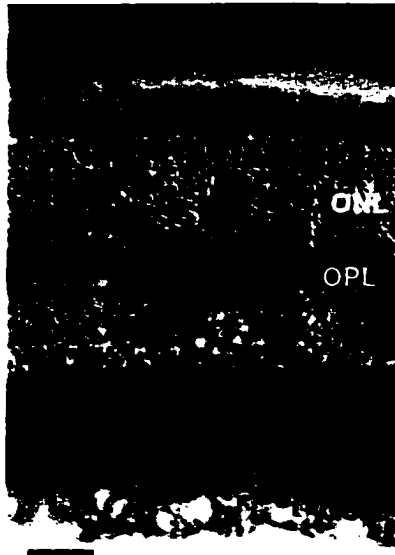
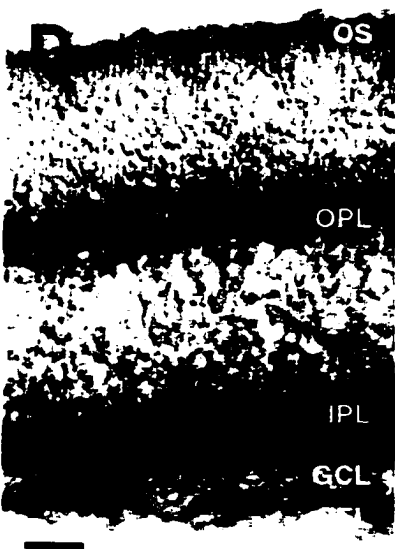
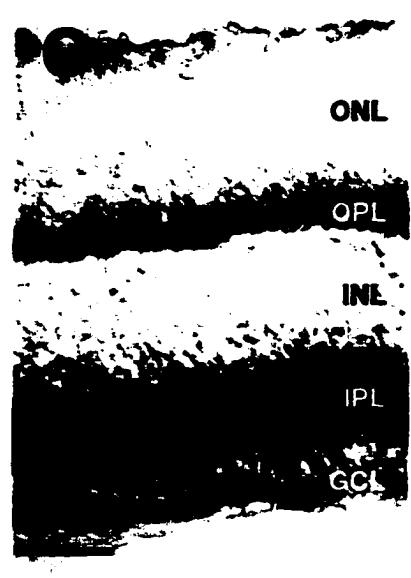
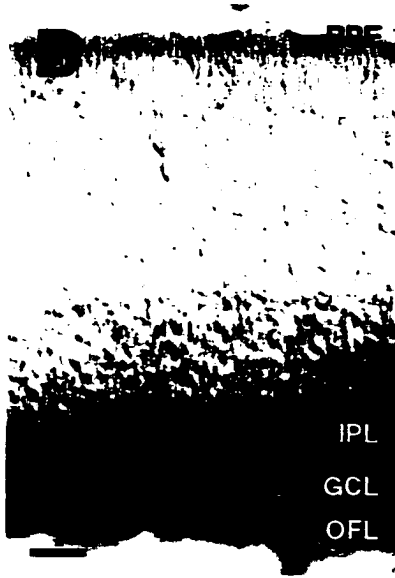
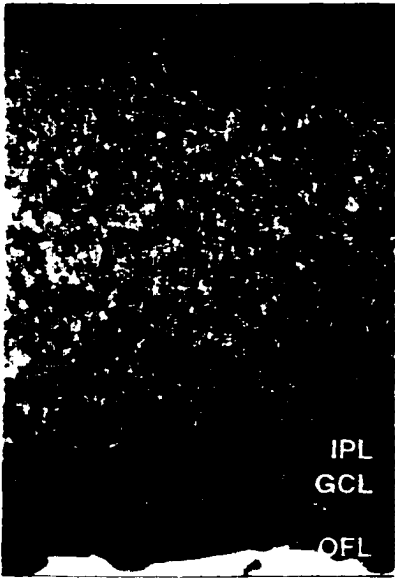


**Figure 5: Syntaxin-immunoreactivity in the postnatal rat retina. At 1PN, Syntaxin -IR was present in the inner retina in the OFL, GCL, differentiating IPL and INL. In addition, cell bodies in the outer retina which appeared to be coalescing in the developing INL was detected (A). In the 5PN rat retina, Syntaxin-IR was detected in the inner retina in the OFL, GCL, IPL, and INL (B). Syntaxin-IR in the 7PN retina was similar to that observed in the 5PN retina (C). By 10PN, Syntaxin-IR was present in the OFL, IPL and OPL, and cells in the inner one-third of the INL. Within the IPL, Syntaxin-IR demarcated the inner and outer regions of the IPL (D). Similar patterns of Syntaxin-IR were observed in the 15PN (E) and adult retina (F). Scale bars; A ,E = 40µm; B, D = 30µm; C, F = 50µm. Abbreviations: ONH, optic nerve head; OFL, optic fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer, OS, outer segments; RPE, retinal pigment epithelium.**



**Figure 6: VAMP-IR in the postnatal rat retina. At 1PN, VAMP -IR was present in the inner retina in the OFL, GCL, differentiating IPL and diffuse throughout the outer three-fourths of the retina. (A). In the 5PN rat retina, VAMP-IR was detected in the inner retina in the OFL, GCL and IPL (B). VAMP-IR in the 7PN retina was localized in the IPL and OPL.**

**Immunoreactivity in the IPL was most intense in sublamina 1, 3, and 5 (C). VAMP-IR in the 10PN retina was also localized to the IPL and OPL. Within the IPL, the most intense VAMP-IR was in the inner one-fourth sublamina, sublamina 5 (D). Similar patterns of VAMP-IR were observed in the 15PN (E) and adult retina (F). Scale bars; A ,E = 40 $\mu$ m; B, D = 30 $\mu$ m; C, F = 50 $\mu$ m.**



SNARE complex proteins in the OPL (Fig. 8). SNAP-25-IR labeled the full extent of the OPL (Fig. 4C-F and Fig. 8A), Syntaxin-IR was observed labeling the inner OPL (Fig. C-F and Fig. 8B) and VAMP-IR was observed in the outer OPL (Fig. 6C-F and Fig. 8C). The triple label (Fig. 8D) shows the inner OPL as purple (red from SNAP-25-IR + Blue from Syntaxin-IR) and the outer OPL as yellow (red from SNAP-25-IR + green from VAMP-IR).

### **Differential expression of SNARE proteins in cellular layers of the developing retina**

In addition to synaptic regions, synaptic terminal proteins in the developing retina are localized in cell bodies after synthesis, before they are transported to developing synaptic zones. Differential expression of SNARE proteins in cellular layers is more obvious in the developing rat retina.

At 1PN, SNAP-25-IR was detected in the GCL, the INL in the region of presumptive amacrine cells, and in cells in the outer retina (Fig. 4A). Syntaxin-IR was detected in cellular layers throughout the extent of the retina in the 1PN rat retina. What appeared to be labeled cell bodies and processes were present throughout the extent of the retina but were most concentrated in the INL in the region of presumptive amacrine cells (Fig. 5A). VAMP-IR was rather diffuse, and detectable in cells throughout the retina (Fig. 6A). In the 5PN rat retina SNAP-25-IR was still detected in the GCL and in cells of the INL (Fig. 4B). The most intensely immunoreactive cells were cholinergic amacrine cells, confirmed by double label analysis with an antibody against choline acetyl-transferase (data not shown). SNAP-25-IR was also detected in the outer one-fourth of the retina. Syntaxin-IR was detected in cells throughout the inner one-half of the 5PN rat retina (Fig. 5B) in a pattern that appeared almost complementary to that of SNAP-25-IR. In contrast, VAMP-IR in the 5PN rat retina was detected only in cells in the GCL (Fig. 6B). Similar labeling was observed in the 7PN retina. In the 10PN rat retina, SNAP-25-IR was diffuse in cells in the inner half of the retina. More

**Figure 7: Differential intensities of SNARE complex protein immunoreactivity in the adult IPL. Density plot profiles taken from images Figures 1-6F (see materials and methods). Relative levels of immunoreactivity for SNAP-25, Syntaxin and VAMP in the IPL of opossum and rat retinas. Plots are from outer to inner IPL and represent a region of IPL approximately 50 x 100  $\mu$ m.**

## Opossum

## Rat

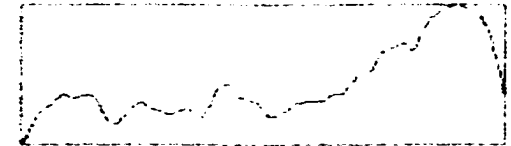
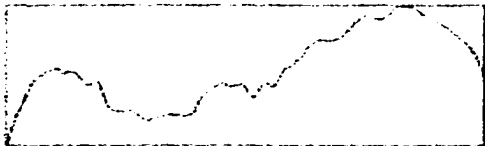
**SNAP-25**



**Syntaxin**



**VAMP**



**Outer  
IPL**

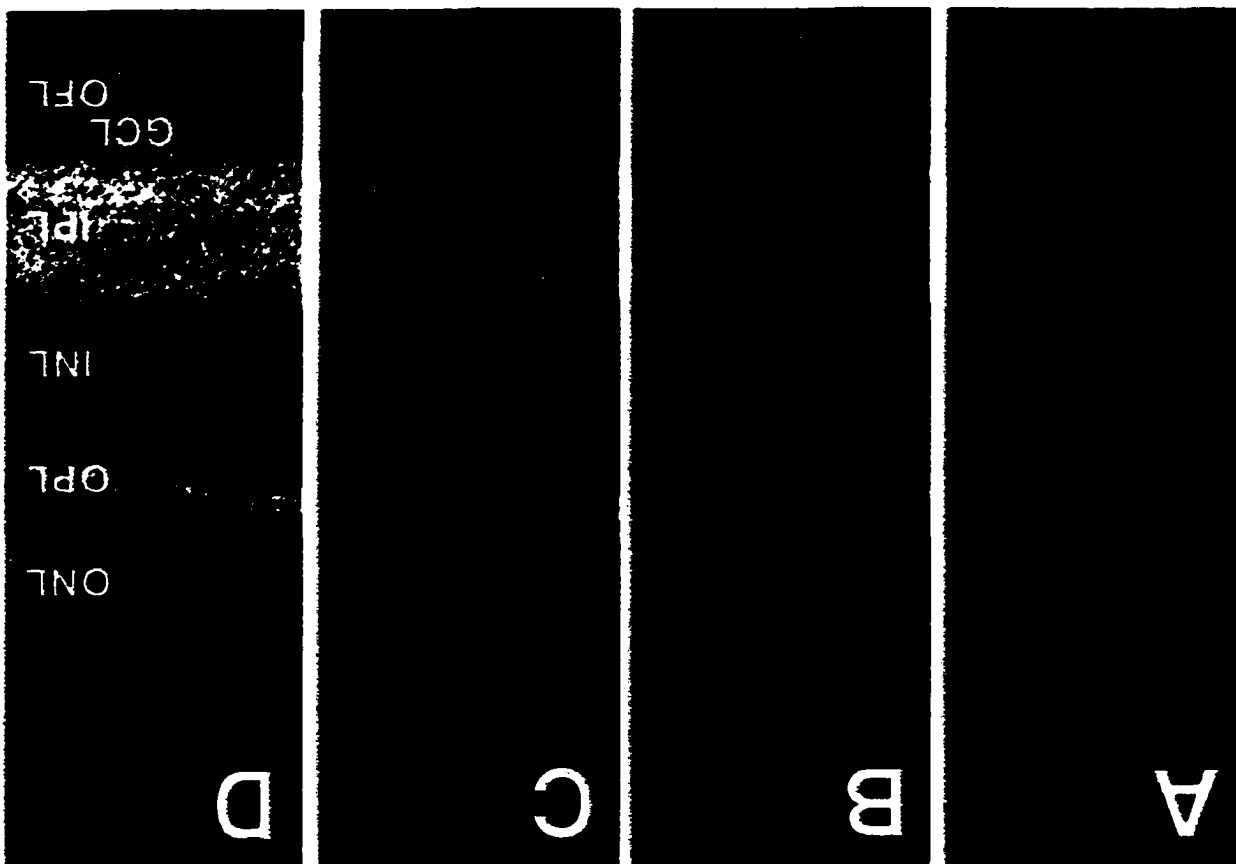
**Inner  
IPL**

**Outer  
IPL**

**Inner  
IPL**

**Figure 8: Localization of SNARE complex proteins in the 15PN rat retina. A section of 15PN rat retina was triple-labeled with antibodies against SNAP-25 (A), Syntaxin (B) and VAMP (C) revealed differential localization of proteins in the OPL. A triple exposure reveals SNAP-25-IR throughout the OPL, the yellow in the outer region of the OPL suggests co-localization of SNAP-25 with VAMP (Red + Green), while the magenta in the inner OPL suggests co-localization of SNAP-25 with Syntaxin (Red + Blue). Co-localization of all three proteins appears as white (see IPL). Abbreviations: OFL, optic fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer, OS, outer segments.**





intense SNAP-25-IR was detectable in the outer half of the INL, and the entire ONL, including the developing outer segments of photoreceptors (Fig 4D). From this age (10PN) through maturity Syntaxin-IR was restricted to cells in the GCL adjacent to the IPL, and cells in the inner one-third of the INL (Fig 5D-F), most likely the amacrine cells. There was no significant VAMP-IR in cellular layers from 7PN through maturity (Fig.6C-F). As the rat retina matured, diffuse SNAP-25-IR was detected in all cellular layers of the rat retina. Much of the immunoreactivity in the ONL was in processes of immunoreactive cells near the outer border of the ONL. In addition, the inner half of the outer segments displayed intense SNAP-25-IR (Fig. 4E,F).

Localization of SNAP-25- and VAMP-IRs was similar in the *Monodelphis* retina (Figs. 1 and 3) when compared to their localization in the rat retina. Syntaxin-IR in the *Monodelphis* retina, however, was not observed in cellular regions until around 25PN, when it was localized to cell bodies in the inner INL (Fig. 2 D-F).

## Discussion

We have characterized the developmental distribution of SNARE complex proteins in the developing mammalian retina. This is the first study to systematically examine the spatial and temporal localization of all three proteins during nervous system development. We observed differential localization of these proteins in both the developing and mature retina of two species. Examination of SNARE complex protein localization in the rat retina allows for comparison with other studies of rat retinal development. For greater temporal resolution of developmental changes in SNARE protein localization, we also examined the retina of the Brazilian opossum, *Monodelphis domestica*. Differential localization of SNAP-25-, Syntaxin- and VAMP-IRs suggests these proteins may play additional roles during development of the retina, independent of SNARE complex formation.

### **SNARE Proteins are differentially expressed in synaptic and cellular layers in the developing mammalian retina.**

The protracted period of development of the *Monodelphis* retina allowed greater resolution of the temporal differences in the onset of SNARE protein expression. In the developing IPL, Syntaxin-IR was detectable prior to significant SNAP-25- or VAMP-IR in that synaptic layer. In the developing OPL, Syntaxin- and SNAP-25-IRs were detectable prior to VAMP-IR. This is consistent with previous studies which have observed developmental functions for SNAP-25 (Osen-Sand *et al.*, 1993; Osen-Sand *et al.*, 1996) and Syntaxin (Igarashi *et al.*, 1996) but not VAMP (Ahnert-Hilger *et al.*, 1996; Osen-Sand *et al.*, 1996) during neurite outgrowth and synaptogenesis. However, it is possible that in this analysis, the temporal differences we observed were due to expression of protein levels of SNAP-25 and VAMP below our detection, rather than a delay in the onset of expression.

During the formation of the IPL, SNAP-25-IR displayed a characteristic pattern. While SNAP-25-IR was observed throughout the IPL, more intense immunoreactivity was localized to sublamina 2 and 4, previously demonstrated to be cholinergic processes of the starburst amacrine cells (West Greenlee *et al.*, 1998). However, this pattern of immunoreactivity was not observed with antibodies to Syntaxin or VAMP suggesting the relatively high levels of SNAP-25 in cholinergic amacrine cells may function independent of Syntaxin and VAMP. In addition, intense SNAP-25-IR in sublamina 2 and 4 was transient during development and was not observed in the adult retina (West Greenlee *et al.*, 1998), further suggesting high levels of SNAP-25 may have a specific function during retinal differentiation. SNAP-25-IR was also localized in the developing outer retina, in the region of developing photoreceptors. SNAP-25-IR in the outer retina was detectable in the 5PN *Monodelphis* retina and at birth in the rat retina. SNAP-25-IR in the outer retina persisted through the period of retinal differentiation, and was observed in the adult retina in the ONL and the inner region of the outer segments. SNAP-25-IR in photoreceptors has also been reported in the adult ferret retina (Karne *et al.*, 1997). The

relatively higher intensity of SNAP-25-IR in these cells, and the absence of immunoreactivity for other SNARE proteins suggests SNAP-25 may be involved in an additional vesicle trafficking event unique to photoreceptors in both the developing and mature retina.

The antibody used to characterize the distribution of Syntaxin, was originally identified as HPC-1, an antibody specific for amacrine cells (Barnstable *et al.*, 1985). In the newborn rat retina, Syntaxin-IR cell bodies were observed in the inner retina, and Syntaxin-IR cells from the outer retina appeared to coalesce around the IPL. Throughout the development of the rat retina, presumptive amacrine cells remained immunoreactive with the antibody. Interestingly, presumptive amacrine cells of the *Monodelphis* retina did not display detectable Syntaxin-IR until around 25PN. Diffuse SNAP-25-IR was detected in the Syntaxin-IR population of presumptive amacrine cells, but no VAMP-IR was detected.

In the 7PN rat retina, VAMP-IR displayed a distinctive tri-laminar pattern. Relatively more intense VAMP-IR was detected in IPL sublamina 1, 3, and 5. This pattern was detected only in the rat, and only at this age. A similar, transient pattern of Synaptophysin-IR, another synaptic vesicle protein (Jahn *et al.*, 1985), has been previously described in the 25PN *Monodelphis* retina (West Greenlee *et al.*, 1996). Similar patterns of immunoreactivity with antibodies against other SNARE proteins were not observed, which may suggest the tri-laminar pattern of VAMP-IR in the IPL represents a transient, high concentration of synaptic vesicles in the regions of more intense VAMP-IR.

Differential localization of SNARE complex proteins in the developing retina may be due to the heterogeneity of cell types and the synapses they make. The differential expression of presynaptic terminal-associated proteins has been observed at functionally different synapses. In the adult retina, biochemically distinct terminals of amacrine cells contain different isoforms of Synapsin, and all isoforms of Synapsin are absent from morphologically distinct ribbon synapses (Mandell *et al.*, 1990). Isoforms of SNARE complex protein Syntaxin are also differentially localized in retinal synapses. Syntaxin-3 is a neuronal isoform which is specific

to, and appears to be the only isoform found at ribbon synapses (Morgans *et al.*, 1996). Multiple isoforms of SNAP-25 (Bark *et al.*, 1995; Bark and Wilson, 1994a) and VAMP have been identified, although there are no published reports about their differential localization in functionally distinct synapses.

**SNARE complex proteins exhibit differential localization in plexiform layers of the mature retina.**

Using density plot profiles we have examined the differential localization of SNARE proteins in the IPL. In the rat IPL, SNAP-25-IR was relatively uniform, but is much more intense in the innermost sublamina, sublamina 5. VAMP-IR had a similar distribution in the adult IPL. The innermost portion of the IPL is the region of rod-bipolar synaptic contacts (see Rodeick, 1998). This may result in a higher synaptic concentration in sublamina 5, or perhaps higher levels of SNAP-25 and VAMP are present in the terminals of rod-bipolar cells. In contrast, Syntaxin-IR was most intense at the inner and outer borders of this synaptic layer. Due to the sparse label in the IPL, it is likely that the Syntaxin-IR in this analysis labels Syntaxin-1, which is absent from ribbon synapses, made by bipolar cells in the IPL (Morgans *et al.*, 1996). Thus, the regions of most intense Syntaxin-IR in the IPL are likely concentrated in conventional-type synapses. It is likely that immunoreactivity for Syntaxin-3, the ribbon specific isoform (Morgans *et al.*, 1996), would be complimentary to Syntaxin-IR we observe with the HPC-1 antibody. We are currently investigating this possibility.

In the outer plexiform layer, SNAP-25-IR appears to label the entire extent of this layer. In contrast, Syntaxin-IR labels only the inner extent of this layer. In contrast, VAMP-IR was observed only in the outer region of the OPL. VAMP is a vesicle associated protein, since horizontal cells have non-vesicular synapses (Schwartz, 1987), the localization of VAMP in the outer region of the OPL, may suggest that the synapses of the photoreceptors are in this outer

region, and the inner region of the OPL is composed principally of non-vesicular horizontal cell synapses.

**Synaptic activity in the developing retina is required for establishment of appropriate circuitry in the visual system.**

Activity-dependent processes during development are important for the establishment of appropriate circuitry in the visual system. Functionally distinct classes of ganglion cells, known as "ON" or "OFF" ganglion cells have their dendrites in the inner or outer region of the IPL respectively. This dendritic organization is dependent on glutamate-mediated activity. Inhibition of glutamate mediated activity in the developing retina disrupts not only the anatomical segregation of these distinct ganglion cell types (Bodnarenko and Chalupa, 1993; Bodnarenko *et al.*, 1995), but also their functional segregation (Bisti *et al.*, 1998). Cholinergic-mediated activity in the developing retina has been demonstrated to be important for the establishment of RGC receptive fields (Sernagor and Grzywacz, 1996). Further, cholinergic mediated synaptic activity is responsible for spontaneous waves of correlated activity (Feller *et al.*, 1996) which are necessary for the segregation of RGC inputs into eye specific layers in the LGN (Penn *et al.*, 1998). It appears that spontaneous activity may be further mediated by GABAergic synaptic transmission as retinal development proceeds (Fisher *et al.*, 1998).

Dynamic differences in localization of SNARE proteins during development may indicate morphological changes taking place within developing synaptic layers. The onset of spontaneous activity in the developing retina appears to precede our detection of laminar organization in the IPL. Spontaneous activity of ganglion cells in rat retina (Galli and Maffei, 1988), and ferret retina (Wong *et al.*, 1993; Wong and Oakley, 1996) is present prior to, or at birth. However, no laminar SNAP-25-IR is detectable until later in development in rat or ferret (Johnson and Reese, personal communication).

The laminar organization of the IPL revealed by SNAP-25-IR appears to precede establishment of on and off bipolar cell terminals. Kahn et al. (1996), used recoverin immunolabeling to examine cone bipolar process stratification in the developing rat IPL. At 5PN, there was no laminar organization of bipolar processes present, however, the laminar pattern of SNAP-25-IR would suggest significant laminar organization is already present in the IPL.

**Expression of SNARE complex proteins is critical for neurite outgrowth and synapse formation.**

SNARE proteins also have critical non-synaptic roles during neural development. Inhibition of SNAP-25 via antisense-oligonucleotides, or via cleavage with botulinum toxin, inhibits neurite outgrowth and synaptogenesis (Osen-Sand *et al.*, 1993; Osen-Sand *et al.*, 1996). Similarly, Igarashi et al. (1996), found that cleaving Syntaxin with botulinum C1 toxin inhibited neurite outgrowth. Further, they inhibited neurite outgrowth using peptides for the VAMP binding site on Syntaxin, suggesting that Syntaxin's role in neurite outgrowth may take place via formation of the SNARE complex. While SNAP-25 and Syntaxin are important during neurite outgrowth, it appears that VAMP is not required for normal neurite outgrowth or synaptogenesis (Ahnert-Hilger *et al.*, 1996; Osen-Sand *et al.*, 1993; Osen-Sand *et al.*, 1996).

SNAP-25 exists as two different isoforms (Bark *et al.*, 1995), which appear to be differentially localized during development and regeneration (Boschert *et al.*, 1996). SNAP-25a, appears to be the more prevalent isoform during development and regeneration, and in NGF-stimulated PC12 cells is localized along the entire extent of axon-like processes (Bark *et al.*, 1995). In contrast, SNAP-25b expression increases steadily during synaptogenesis, and when expressed in PC12 cells, is localized to terminals (Bark *et al.*, 1995). Thus, differential expression of SNAP-25 isoforms may account for differential functions for the protein in cells. The SNAP-25 antibodies used in this analysis recognize both isoforms of SNAP-25, so it is

not known which isoform contributes to the relatively high levels of SNAP-25-IR in cholinergic amacrine cells or cells of the outer retina.

Though SNAP-25 and Syntaxin have both been demonstrated to be critical for synaptic transmission and neurite outgrowth, they appear to be differentially localized within the developing and mature inner plexiform layer. This may be due to the fact that SNAP-25 appears to be localized to both ribbon and conventional synapses (Ullrich and Sudhof, 1994), while the isoform of Syntaxin likely recognized by our antibody is absent from ribbon synapses. However, in cholinergic amacrine cells, which form conventional synapses, high levels of SNAP-25-IR are not accompanied by high levels of Syntaxin-IR. It is not clear whether SNAP-25's role during neurite outgrowth is dependent on Syntaxin. Therefore, it is possible that high levels of SNAP-25 expression during development are required for extensive outgrowth and/or remodeling of cholinergic terminals via non-Syntaxin dependent mechanisms.

We have observed spatial and temporal differences in the localization of the SNARE complex proteins SNAP-25, Syntaxin, and VAMP, in the developing mammalian retina. This is the first analysis to systematically examine the localization of these proteins during retinal development. SNARE complex proteins exhibit differential localization in the developing and mature retina. Thus, in addition to their function during regulated release of neurotransmitter, SNAP-25, Syntaxin, and VAMP may have additional, independent roles during vesicle trafficking in the mammalian retina.

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## GENERAL CONCLUSIONS

We have characterized the localization of SNARE complex and other presynaptic terminal proteins during development and differentiation of the mammalian retina. These proteins exhibited dynamic, differential patterns of immunoreactivity as the retina matured. We described the developmental distribution of immunoreactivity for proteins of the synaptic vesicle (Rab3A, Synaptophysin (p38), Synaptotagmin (p65), Synaptobrevin/VAMP) and presynaptic membrane (SNAP-25, Syntaxin). In addition, we have described the dynamic changes observed in levels of SNAP-25 during differentiation of a crucial cell type in the developing retina, the cholinergic amacrine cells.

In general, there was a shift of protein localization from cellular layers where it is synthesized in the soma of neurons early in development, to plexiform layers, presumably to synaptic terminals as they differentiated. However, there were many cases in which there were subtle, but consistent differences in the spatial and temporal patterns of immunoreactivity for different synaptic proteins.

We observed temporal differences in the onset of detectable immunoreactivity for synaptic proteins during development. This is particularly striking when comparing the patterns of immunoreactivity for the four synaptic vesicle associated proteins. Immunoreactivity for synaptic vesicle proteins Synaptotagmin (p65) and Rab3A was detected earlier (5PN in *Monodelphis*), and was localized in the IPL in the 10PN opossum retina, while Synaptophysin (p38)- and Synaptobrevin/VAMP-IRs were not localized to the IPL until 15PN. Since all four proteins are found associated with synaptic vesicles, one might expect similar spatial and temporal patterns of expression. The earlier detectability of Synaptotagmin- and Rab3A-IRs may suggest they have a developmental vesicle trafficking role in addition to their role during transmitter release.



Temporal differences in the expression of SNARE complex proteins was also observed. In the differentiation of the IPL of *Monodelphis* retina, significant immunoreactivity for SNAP-25 and Syntaxin preceded VAMP-IR. This is consistent with reports that SNAP-25 and Syntaxin have a developmental role during neurite outgrowth (Igarashi *et al.*, 1996; Osen-Sand *et al.*, 1993; Osen-Sand *et al.*, 1996), while VAMP does not (Osen-Sand *et al.*, 1996).

In addition we observed a distinctive, transient pattern of SNAP-25-IR, which was not observed with antibodies against any other presynaptic terminal-associated protein. Around 15PN in the *Monodelphis* retina, relatively higher levels of SNAP-25-IR were present in the somata and processes of cholinergic amacrine cells. These cells are the only cholinergic cells in the retina and are responsible for the correlated spontaneous activity which is necessary for the establishment of appropriate circuitry in the retina (Sernagor and Grzywacz, 1996) and the rest of the visual system (Penn *et al.*, 1998). The onset of high levels of SNAP-25-IR coincided with the detectability of choline acetyl transferase-IR in these same cells (West Greenlee *et al.*, 1998). We observed transient, high levels of SNAP-25 in cholinergic amacrine cells prior to eye opening, a time when spontaneous retinal waves are likely present. High levels of SNAP-25 are also present in cholinergic cells of the developing ferret retina (Johnson and Reese, personal communication), an animal in which spontaneous retinal waves have been well characterized (Feller *et al.*, 1996; Meister *et al.*, 1991; Wong, 1993). Perhaps the high levels of SNAP-25 in these cells contribute to their involvement in spontaneous retinal activity. However, if high levels of SNAP-25 were involved exclusively in transmitter release, we might expect to see similar patterns of immunoreactivity for Syntaxin and Synaptobrevin/VAMP. These proteins, though present in the IPL at this age, do not appear to be at higher levels in cholinergic cells. Alternatively, high levels of SNAP-25-IR may be due to extensive growth or remodeling of cholinergic processes during establishment of laminar organization within the IPL. There are two isoforms of SNAP-25 (indistinguishable immunohistochemically). Of these isoforms it has been proposed that one (SNAP-25a) may be

involved in neurite outgrowth and the other (SNAP-25b) may function predominately during transmitter release (Bark *et al.*, 1995). If one isoform contributes predominantly to the transient high levels of SNAP-25 in developing cholinergic amacrine cells, it may provide a clue as to the functional role for the dynamics of this protein expression. We are currently investigating this possibility using in situ hybridization to differentiate between SNAP-25 isoforms at the mRNA level.

Further studies which compare the development of laminar organization in the IPL with changes in presynaptic terminal protein localization may reveal a developmental relevance for the observed dynamic differences. Laminar organization was originally defined in the mature retina histochemically based on processes of amacrine cells with different neurotransmitter phenotypes (Karten and Brecha, 1983; Marc, 1986). Laminar organization can also be defined by the termination of bipolar cell terminals from bipolar cells into ON or OFF sublamina in the inner 3/5 and outer 2/5 respectively (Kahn *et al.*, 1997), or by restriction of ON or OFF RGC dendrites to the similar location (Bodnarenko and Chalupa, 1993). We have observed laminar differences in the localization of presynaptic terminal proteins in the developing retina. The most striking is certainly the relatively high levels of SNAP-25-IR in sublamina 2 and 4 in the 25PN opossum retina. Interestingly, at this same age, synaptic vesicle protein Synaptophysin (p38) is relatively most intense in sublamina 1, 3, and 5. In the developing rat retina at 7PN (similar in maturity to a opossum 25PN retina) VAMP-IR displays a very similar pattern to Synaptophysin-IR (p38) in the 25PN opossum retina, that is, relatively more intense immunoreactivity in sublamina 1, 3, and 5.

Presynaptic terminal proteins have differential localization in the mature as well as developing IPL. In section three we provide a comparison of differential intensity of immunoreactivity between SNARE complex proteins. For example, sublamina 5 has the most intense immunoreactivity for both VAMP and SNAP-25. Differential intensity of immunoreactivity for these proteins may be the result of differing synaptic density, that is

sublamina 5 may have a higher concentration of synaptic contacts than the other sublamina. Syntaxin-IR is most intense at the inner and outermost borders of the IPL. This may be because these regions have the highest concentration of conventional synapses, while the middle of this layer has more ribbon synapses that express a different isoform of Syntaxin, Syntaxin 3 (Morgans *et al.*, 1996).

SNARE complex proteins have differential localization in the OPL as well. SNAP-25-IR is observed through the extent of the OPL. VAMP-IR, however, was only observed in the outer half of the OPL. Faint Syntaxin-IR was detected in the inner half of the OPL where VAMP-IR was absent. However, this Syntaxin antibody likely does not recognize Syntaxin 3, the isoform present at the ribbon synapse-terminals of photoreceptors (Morgans *et al.*, 1996). We are not aware of any studies which have described a laminar organization of the OPL. However, our results suggests that there is a laminar organization to this layer. Since VAMP is a synaptic vesicle protein, we suggest it is present in the vesicles at photoreceptor terminals, and is absent from terminals of horizontal cells which make non-vesicular synapses (Schwartz, 1987). The inner region of the OPL is a plausible region for terminals of horizontal cells, because their cell bodies are in the inner nuclear layer, adjacent to the inner OPL. Thus, perhaps the light Syntaxin-IR we observe, which appears to be present in the inner region of the OPL lacking Synaptobrevin-IR, is also present in terminals of horizontal cells. This differential localization of SNARE complex proteins was not observed until a triple-label analysis for all three proteins in one tissue section was done. It may be useful to compare expression of other vesicle associated proteins (i.e. p38, Synaptotagmin) in a double label analysis with SNAP-25 so see if they too are restricted to the outer half of the OPL.

Immunoreactivity for synaptic terminal proteins in cellular layers is common during development but is much less common in the mature retina. However, significant SNAP-25-IR has been observed in cell bodies of photoreceptors in the mature retina (Karne *et al.*, 1997). SNAP-25-IR is observed in the outer retina quite early in retinal development. This SNAP-25-

IR co-localizes with immunoreactivity for antibodies against recoverin (Bookland and Sakaguchi personal communication), suggesting that even early in development SNAP-25-IR in the outer retina is present in developing photoreceptors. In the mature retina, SNAP-25-IR is observed in cell bodies of photoreceptors and in the inner region of the outer segments. While immunoreactivity for other presynaptic terminal associated proteins is observed in the outer plexiform layer, presumably in photoreceptor terminals, no other protein appears to have such an extensive localization in these cells. Thus, it is possible that SNAP-25 functions in an additional SNARE-independent vesicle trafficking event unique to photoreceptors.

Finally, characterization of the developmental expression of presynaptic terminal associated proteins, has given us an assay to measure appropriate retinal development. By comparing relative onset and intensity of protein expression, we can reliably use this standard when assessing the potential effects of any manipulation on retinal development.

We have described differences in the spatial and temporal localization of presynaptic terminal associated proteins during development of the mammalian retina. The use of the Brazilian opossum retina as our primary model system has allowed us to resolve subtle temporal differences in the localization of some proteins which may have been overlooked had we used a more conventional model system with a much less protracted period of development. Differential localization during development of proteins which one would expect to be localized in the mature retina (i.e. proteins of the synaptic vesicle or SNARE complex proteins) may suggest a role for some presynaptic terminal proteins in vesicle trafficking events during process outgrowth and synaptogenesis.

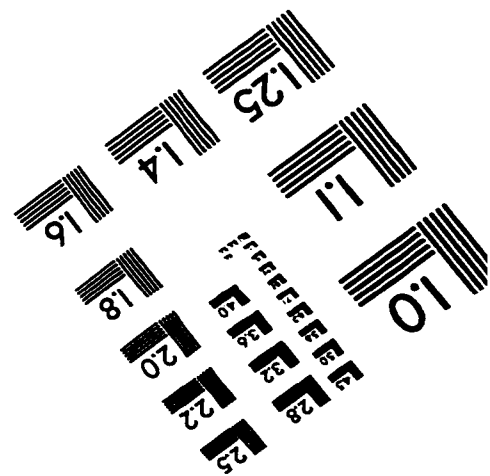
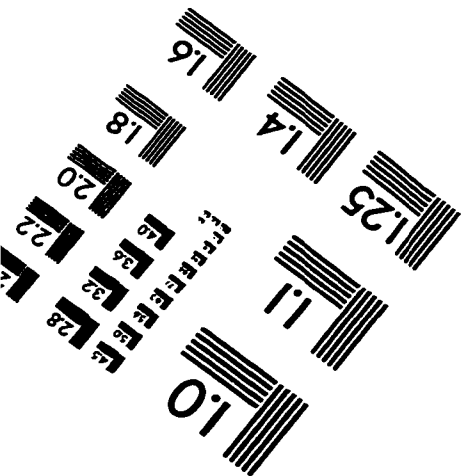
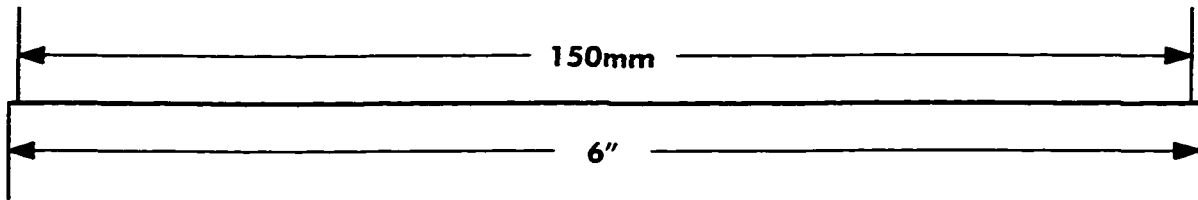
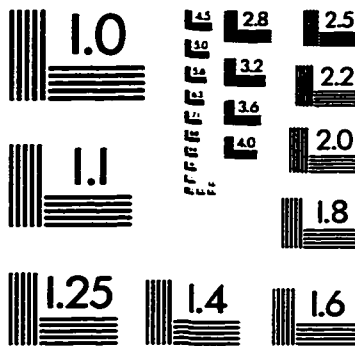
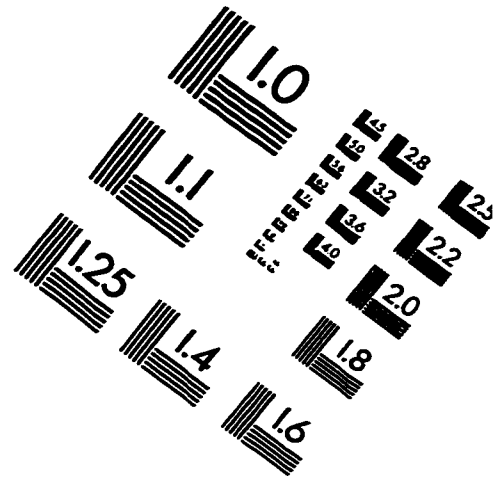
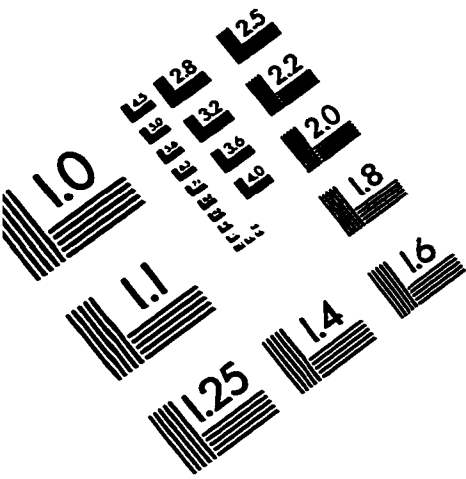
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